THE VIRUS-HOST CELL RELATIONSHIP

by

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INTRODUCTION

Many factors may control whether or not a cell is susceptible to infection by a given virus, even assuming the virus is not defective. The viral replication cycle may break down at any one of a number of places, due to some form of inadequacy of the cell. The earliest stage at which such virus-host cell incompatibility can arise is at initial virus adsorption.

The initial attachment of a bacteriophage to a susceptible bacterium is known to involve union between complementary surfaces on the tail of the phage and the superficial layers of the bacterium (Cruickshank, 1965). This process is so highly selective that the surface components of the bacterial cell exhibit greater specificity in this respect than they do in serological reactions. Bacteria lacking the particular surface configuration are insusceptible to infection with the corresponding bacteriophage. The chemical nature of the phage receptor areas on the bacterial cell wall has been found to be mucopeptide, although in some cases it has been identified as lipopolysaccharide (Weidel et al., 1954).

Myxoviruses are known to attach to specific receptor areas on the surfaces of cells of the respiratory tract and on red blood cells (Gottschalk, 1959). These latter receptors appear to be composed of neuraminic acid-containing mucoproteins. The specificity of such receptor sites is demonstrated by the

receptor gradient found in myxovirus adsorption to fowl red cells. Virus elutes from cells after prolonged contact at room temperature due to the action of the virus enzyme, neuraminidase, which destroys the receptor. Only the other myxoviruses placed lower on the receptor gradient, and presumably with progressively lower specificities for receptor configuration, can then adsorb to such cells.

Holland and his co-workers (e.g. Holland, 1961) have demonstrated conclusively that specific receptor areas for enteroviruses exist on the surfaces of susceptible mammalian cells, but are absent on cells insusceptible to infection by these viruses. They showed that while cells from hosts or from tissues insusceptible to poliovirus were unable to support viral replication when exposed to complete infectious virus particles, they could support virus growth when exposed to infectious viral RNA (Holland et al., 1959). By infecting with viral RNA the 'specific receptor' mode of virus entry to the cell had been by-passed. Alteration of species and tissue specificity of poliovirus has been demonstrated (Cords and Holland, 1964) by inclusion of infectious viral RNA within the protein capsid of coxsackie B_1 virus. From these and related findings they have explained the tissue tropism occurring in poliovirus infections on the basis of the presence or absence of these specific virus adsorption

areas on cell surfaces.

An attempt was made to characterise enterovirus receptors. Unlike the mucopeptide receptors for bacteriophages and the mucoprotein receptors for myxoviruses, receptors for enteroviruses were found to consist of a lipoprotein mosaic (Holland and McLaren, 1961).

The existence of specific receptors in these studies was indicated by demonstrating loss of viral infectivity when virus was mixed with a homogenate of susceptible cells. Admixture resulted in attachment of virus to receptor material with subsequent reversible eclipse of infectivity (Holland, 1962). Attachment of virus to microsomal material proceeded no further, but attachment of virus to cell membrane fractions led ultimately to an irreversible eclipse of infectivity.

This work was confirmed by similar results obtained by Kunin (1962) on receptors for coxsackie B_1 and B_3 viruses.

Quersin-Thiry and Nihoul (1961) demonstrated a similar type of virus inactivation by cell homogenates. Their work confirmed the findings of Holland and his colleagues in that polio and coxsackie viruses were inactivated by homogenates of susceptible but not of insusceptible cells. They demonstrated a similar inactivation of Newcastle disease virus, of western

equine encephalomyelitis virus and of vaccinia virus. Furthermore, they postulated the existence of at least three different types of inactivators in HeLa cell homogenates. The first combined with any of the three types of poliovirus and was sensitive to trypsin. The second combined with western equine encephalomyelitis virus and was sensitive to lipase but not to trypsin. The third combined with Newcastle disease virus and appeared similar to known receptors for the myxovirus group. The material which inactivated coxsackie B_1 and B_4 viruses appeared similar to the inactivator of Newcastle disease virus.

These results suggested that it might be possible to differentiate the major groups of viruses according to the nature of the site of attachment on mammalian cells. The fact that there may be different receptor sites for some groups of viruses was not considered surprising since there are major serological distinctions between the virus groups. It was interesting however, to find that the receptor areas were of different chemical types, e.g. mucoprotein for myxoviruses and lipoprotein for enteroviruses.

The original purpose of the work contained in Part I of this thesis was to investigate further the report of Quersin-Thiry (1961) that material existed in tissue cell homogenates which was able to inactivate vaccinia virus, After this, the object was to

elucidate the relationship between inactivation of virus by cell homogenates and the existence of specific virus receptors. Finally, it was hoped to investigate the specificity of receptors for different members of the poxvirus group and if possible to determine the nature of the receptor site.

However, early experiments showed no specific inactivation of vaccinia virus by homogenates of susceptible cells: a finding which was confirmed repeatedly. The result of incubation of virus in the presence of cell homogenate was a consistent increase in the titre of virus, as compared with controls. Further work was then carried out to elucidate this finding. The increase in titre may have been due to stabilization of the virus against some non-specific inactivation, such as by heat or adverse effect of diluting fluid. The investigation was therefore extended to cover the stability of the infectivity of vaccinia virus to various physical and chemical treatments.

PART I

Interaction between Cellular Homogenates and Vaccinia Virus

MATERIALS AND METHODS

VIRUSES

1. Vaccinia virus

a) The lapinised Lister strain had been passed many times in this laboratory through rabbits and then through eggs. This was used as seed to prepare a large batch of virus for these studies.

b) A second strain of vaccinia was obtained from Evans Medical Company, Speke, Liverpool, through the courtesy of Dr D. Hobson. This virus is genetically distinct from the Lister strain; it produces haemorrhagic pocks on the chorioallantoic membrane of fertile eggs and forms plaques on RK_{13} monolayers more slowly than does the Lister strain.

2. Cowpox virus

The strain used was the KP strain originally obtained from Dr F.O. MacCallum and subsequently passaged in eggs in this laboratory for 12 years.

3. Poliovirus type 1

The Mahoney strain of type 1 poliovirus was used. This virus had been obtained from the Central Public Health Laboratory, Colindale, and subsequently passaged in this laboratory for many years in HeLa cells to give a crude freeze-thaw lysate.

CELLS

1. Rabbit kidney. Two strains of a rabbit kidney cell line were obtained from Dr J. McKay, Moredun Research Institute, Edinburgh. These are uncloned lines of rabbit kidney epithelial cells. The first was designated the RK-13 line and originated from Glaxo Laboratories Ltd., Greenford, England. The second was designated 6RK-13. The lines probably originated from the same source and appeared identical in many characteristics, e.g. morphology, growth requirements, and plaque type produced by infection with several viruses. They differed slightly in the ease with which they were detached from glass by a versene-trypsin mixture. The second was used extensively in this work and will be referred to throughout as the RK_{13} cell line. The first will be differentiated here as the Glax. RK_{13} line.

2. HeLa. A subculture of this cell line was kindly supplied by Prof. N. Grist, Ruchill Hospital, Glasgow. These are malignant epithelial cells of human origin.

3. HEp-2. A subculture of this cell line was obtained from the Institute of Virology, Glasgow. This also is a line of malignant epithelial cells of human origin.

MEDIA

1. The rabbit kidney cell lines were propagated in Glaxo tissue culture medium 199 supplemented with 10 per cent. calf serum.

2. HeLa cells were propagated in Hanks balanced salt solution (Hanks BSS) containing 0.25 per cent. Difco lactalbumin hydrolysate, 0.044 per cent. sodium bicarbonate and 20 per cent. human serum.

3. HEp-2 cells were propagated in Burroughs Wellcome Eagle's tissue culture medium containing 10 per cent. Difco tryptose phosphate broth, 0.044 per cent. sodium bicarbonate and 10 per cent. calf serum.

4. The medium used for the preparation of cell monolayers for experimental purposes and for use as routine diluent, was a modified form of the medium described by Appleyard and Westwood (1964). It consisted of an Earle's balanced salt solution containing 0.05 per cent. galactose, 0.25 per cent. Difco lactalbumin hydrolysate and 10 per cent. Difco tryptose phosphate broth; but contained no glucose, no sodium bicarbonate and no serum. Cultures could be incubated in this medium in open vessels without the necessity of enriching the atmosphere with added carbon dioxide. The pH remained constant due to the presence of the phosphate buffer and the absence of glucose.

All sera used were sterilized by Seitz filtration and inactivated by heating for 30 minutes at 56°C. All media contained penicillin and streptomycin to a final concentration of 100 units per ml and 100 µg per ml respectively.

Overlay media were developed during the course of this work and are described on pages 26-30.

GROWTH OF CELLS

Growth of cells and conditions of incubation were as described in Part II. Preparation of cell monolayers for experimental purposes was also as described in Part II with one difference. Cultures were sown in the serum-free galactose medium (SF gal) and therefore could be incubated without enriching the atmosphere with carbon dioxide until the overlay media were added.

PRESERVATION OF CELL STOCKS

Cell stocks were preserved in glycerol at -65°C, see Part II.

PREPARATION AND STORAGE OF VIRUS STOCKS

1. Vaccinia virus

a) An aliquot of the Lister strain of vaccinia was used as seed to prepare a large batch of virus by propagation in vitro in HeLa cells. Virus was harvested after 24 hours growth by disrupting the cells with an MSE ultra-sonic vibrator operating at 20 Kc per second for 5 minutes. The suspension was purified using a modified version of the technique described by Joklik (1962a). The ultra-sonicate containing the virus was centrifuged for 10 minutes at 750 g to remove large pieces of cell debris; the supernate centrifuged for 30 minutes at 15,000 g in a Spinco angle head rotor 21; the virus deposit was re-suspended in a small volume of phosphate buffered saline at pH 7.4 and layered on top of a 30 per cent. sucrose solution contained in a suitable Spinco tube. This was then centrifuged for 80 minutes at 23,000 g in the SW 39L Spinco head. The following fractions resulted: an almost clear top layer, material at the buffer sucrose interface which would not spin into the sucrose, an opalescent sucrose phase and finally a pellet which contained most of the virus. The pellet was resuspended in phosphate buffered saline at pH 7.4 and subjected to ultra-sonic treatment for 2 minutes to give a preparation of titre approximately 10^7 pl.f.u.

per ml. This was dispensed in 1 ml volumes in bijoux bottles and stored at -65°C. One bottle was used as starting material for one experiment.

Upon examination under the electron microscope a sample of the above preparation stained with phosphotungstic acid appeared free from cellular material. Virus occurred about 50 per cent. singly and 50 per cent. in clumps of 2-7 particles. This preparation was considered purified for the purpose of this investigation, i.e. freed from any "cell receptor" material.

b) The Evans strain of vaccinia was propagated in RK_{13} cells and carried through the purification procedure described for the Lister strain. The final preparation, having a titre of approximately 10^7 pl.f.u. per ml, was similarly dispensed and stored.

2. Cowpox virus

This virus was propagated in RK_{13} cells. It was purified, dispensed and stored as were the two vaccinia preparations. The titre of the final preparation was 4×10^7 pl.f.u. per ml.

3. Poliovirus type 1

This stock was prepared by infecting HEp-2 cells in Roux bottles at unknown multiplicity, incubating at 37°C and harvesting when widespread cytopathic effect

was observed. Virus was liberated from cells by three cycles of freezing and thawing and titrated in tube cultures of HEp-2 cells. The end-point of the titration was calculated using the formula of Reed and Meunch (1938).

PREPARATION OF TISSUE CELL HOMOGENATES

In preparing cell homogenates, detachment of the cells from the glass by trypsin was avoided to obviate any digestion of specific protein configurations on the cell surface. The method usually followed was that described by Quersin-Thiry (1961). Cell monolayers were washed twice with Hanks BSS at pH 7.4, and the cells removed from the glass by shaking with glass beads. The cells were deposited by centrifugation, washed a further twice and finally suspended in Hanks BSS to give a concentration of 10^7 cells per ml. Homogenates were prepared by subjecting cells to seven cycles of freezing and thawing. They were then dispensed in bijoux bottles and stored at -65°C.

Homogenates of both rabbit kidney cell lines, of HeLa cells and of HEp-2 cells were prepared in this way. One batch of RK_{13} homogenates was prepared according to the method of Holland and McLaren (1961) using 0.25 M sucrose as the suspending fluid, chilling the washed cells to 0°C in an ice-alcohol bath, and disrupting using a Potter-Elvejhem grinder with a teflon shaft.

One batch of RK_{13} cells was fractionated using the method of Holland and McLaren (1961). This involved differential centrifugation in 0.25 M sucrose; and applying forces of 800 g to sediment the nuclear

fraction, of 7000 g to sediment the mitochondrial fraction, of 38,000 g to sediment the microsomal fraction and of 105,000 g to sediment the post-microsomal fraction. The 'cell sap' fraction was left in the supernate. All fractions were finally suspended in 0.15 M sodium chloride and stored at -65°C.

Other reagents used in further treatment of homogenates are listed below.

Difco trypsin 1:250 was obtained from Difco Laboratories Inc., Detroit, Michigan. A 1 per cent. solution was prepared in Hanks BSS.

Lipase and sodium metaperiodate were obtained from L. Light and Co., Ltd., Colnbrook, Bucks. A 1 per cent. solution of lipase was prepared in veronal buffered saline and stored at -30°C. A $\frac{5}{4}.10^{-3}$ M solution of sodium metaperiodate was prepared in 0.85 per cent. saline and stored at 4°C.

Glycerol, 'Analar', was obtained from British Drug Houses Ltd., Poole, England. This was prepared as a 5.10^{-3} M solution in 0.85 per cent. saline and stored at 4°C.

STANDARD TITRATION TECHNIQUES

1. Plaque Titration Technique

The titration technique for poxviruses was investigated in some detail as reported on pages 23-31.

That described below was selected as the standard technique.

Monolayers of RK_{13} cells were prepared in glass petri dishes of 6 cm diameter by sowing 4 x 10^6 cells in 5 ml of galactose medium supplemented with 10 per cent. calf serum. Cultures were incubated at 37°C overnight. Before inoculation with virus, cultures were drained, and 1 ml of virus suspension diluted in SF gal medium then added to each petri dish. The cultures were incubated for 3 hours at 37°C with occasional gentle rocking to allow virus to adsorb to cells. Overlay medium containing 0.75 per cent. Methocel was then added, 10 ml per petri dish, without removing the residual inoculum fluid. Methocel was a preparation of methylcellulose obtained from L. Light and Co., Ltd., Colnbrook, Bucks.

Cultures were incubated for 48 hours in an atmosphere enriched with 5 per cent. carbon dioxide to allow plaques to develop. To count plaques, the Methocel medium was removed and the cell sheets were stained with a 0.1 per cent. solution of methyl violet in physiological saline.

2. Tube Titration Technique

This was as described in Part II but the virus diluting fluid was SF gal and not skim milk.

VIRUS STABILITY EXPERIMENTS

Tests were always carried out in pyrex vessels. These were usually 25 ml conical flasks with cotton-wool plugs, but sometimes 15 x 1.5 cm test-tubes with rubber bungs were used. The virus preparation, 0.1 ml, was added to 0.9 ml of diluent under test in the reaction vessel to give a virus concentration of 10^4 pl.f.u. per ml. This was then incubated in a water bath at the required temperature usually for 1 hour although this reaction time was varied. Whether incubation was stationary or with continued agitation will be stated in the results. After the test period the sample was withdrawn, diluted in SF gal and titrated on RK_{13} monolayers by the standard technique.

In some cases the diluent under test was a cellular homogenate of standard concentration, and in some cases a suspension of viable cells.

The effect of ultra-sonic treatment on virus preparations was tested using an MSE probe-type ultra-sonic disintegrator running at maximum frequency (20 Kc per second). The sample under test was contained in a 10 ml standard MSE ultra-sonic tube which was pyrex, thick-walled and round-bottomed. The tube was fitted with a rubber cap through which the probe entered thus producing a hermetically sealed system. During ultra-

sonic treatment the tube was surrounded by an ice bath. Samples were of 5 ml volume and contained virus at a concentration of 10^6 pl.f.u. per ml.

In experiments designed to determine the effect of increased surface area of the reaction vessel on virus survival two methods were used. One was to agitate the vessel throughout the test period and thus increase the area of contact of the sample. This was done using a water bath fitted with a platform which could be agitated mechanically. The other method used was to increase the total glass surface area by the introduction of small glass beads, e.g. Ballotini beads, to the reaction vessel.

PART I

Interaction between Cellular Homogenates and Vaccinia Virus

RESULTS

DEVELOPMENT OF A PLAQUE TITRATION TECHNIQUE FOR POXVIRUSES

1. Selection of Cell Line

Titrations of the Lister strain of vaccinia virus were carried out on HeLa and on RK_{13} monolayers. In these experiments the inoculum volume was 1 ml and the adsorption period was 3 hours at 37°C. The overlay was the appropriate medium for the cells containing 0.75 per cent. Methocel. On HeLa monolayers plaques of less than 1 mm in diameter were formed in 68 hours, while on RK_{13} monolayers plaques of about 1 mm in diameter were formed in 44 hours. Plaque counts obtained on RK_{13} monolayers were consistently greater, of the order of 20 per cent. greater, than on corresponding HeLa cultures. Because of this slightly increased sensitivity and the quicker formation of plaques, the RK_{13} was selected as the standard cell line for all titrations in preference to the HeLa line.

Both the Evans strain of vaccinia and the KP cowpox strain formed smaller plaques than the Lister vaccinia. The former two therefore, were routinely incubated for 68 hours when plaques of about 2 mm in diameter were formed and the latter for 44 hours.

2. Length of Virus Adsorption Period

For reasons given below, medium containing

0.75 per cent. Methocel was used as the overlay to prevent secondary plaque formation. The effect on virus titre of duration of the virus adsorption period before addition of overlay, was investigated by adding Methocel containing overlays to cultures at various intervals post-inoculation. Residual inoculum fluid was not removed before addition of the overlay.

Results obtained from such an experiment are shown in Table 1.

Table 1. Kinetics of adsorption of vaccinia virus to RK_{13} monolayers.

Adsorption period (in hours)	Number of plaques formed per culture
1	102
2	169
3	216
4	227
5	295
6	370

As shown in Table 1 adsorption of virus increases with time before the addition of overlay. It was decided to select 3 hours at 37°C as standard adsorption conditions since a considerable proportion of virus is adsorbed in this time. A 5 or 6 hour adsorption period would be complicated by the termination of viral eclipse

and the possibility of subsequent secondary infection. Although total adsorption was not achieved in 3 hours, the fraction adsorbed should remain constant in a series of titrations if standard techniques are employed throughout.

3. Overlay Media

Since incubation in normal liquid medium allows the formation of secondary plaques, incubation under three overlays was compared. Those investigated were, an agar overlay (Dulbecco and Vogt, 1954), a methylcellulose overlay (Russell, 1962), and a liquid overlay incorporating homologous antiserum. For the agar and methylcellulose (Methocel) overlays the adsorption period selected was 3 hours. An antiserum overlay was not added until 16 hours post-infection according to the method of Postlethwaite (1960). Complications arise if antiserum is added soon after inoculation because of neutralisation of extracellular virus.

The agar overlay consisted of Difco noble agar at a final concentration of 1.2 per cent. in a 199 calf serum medium. After incubation for plaque formation the agar disc was carefully removed without damaging the cell sheet and the cultures stained with a 0.1 per cent. solution of methyl violet in saline. Alternatively it was possible to stain plaques by applying a second agar overlay containing neutral red and further incubating for a few hours. Plaques thus stained were not so clearly visible as those stained by the first method.

The effect of a range of Methocel concentrations was tested. Firstly, the preparation of stock Methocel

will be described. Because of the high viscosity of concentrated solutions of this substance it was not possible to prepare a solution containing more than 5 per cent. w/v of the dry powder. To 'wet' the powder 5 gm of dry Methocel powder was stirred in 50 ml of distilled water heated to 90°C. This was then cooled to 4°C when it became more liquid, and a further 50 ml of distilled water at 4°C added. The resultant preparation was dispensed in 50 ml volumes and autoclaved, 115 lb for 15 minutes, and stored at 4°C prior to use.

The effect of a range of Methocel concentrations was tested and the results obtained are shown in Table 2.

Table 2. Effect of concentration of Methocel in the overlay medium in vaccinia plaque titrations.

Methocel concentration (per cent.)	Number of plaques formed per culture	
	A	B
0.75	75	271
1.00	65	273
1.25	69	264
1.50	58	227

A and B are repeat experiments.

When Methocel overlays were used great care was

taken to avoid any disturbance of the cultures during incubation since these media never became solid but prevented secondary plaque formation by virtue of their high viscosity. From the results shown in Table 2 it was decided to use 0.75 per cent. Methocel in 5 per cent. calf serum 199 routinely as overlay medium. Higher concentrations of Methocel were more difficult to work with because of the increased viscosity and moreover did not appear to cause any further reduction in secondary plaque formation. When the Methocel concentration was increased to 1.5 per cent. it exerted a deleterious effect on the cells and caused a reduction in plaque size.

The use of an antiserum overlay was investigated by incorporating different concentrations of rabbit anti-vaccinial antiserum into the standard growth medium. According to the method of Appleyard and Westwood (1964) a 3 ml inoculum and an adsorption period of 16 hours at 37°C were used.

Results obtained are shown in Table 3.

Table 3. Effect of concentration of homologous antiserum in the overlay medium in vaccinia plaque titrations.

Antiserum dilution	Number of plaques formed per culture
10^{-2}	122 †
$\frac{1}{2}.10^{-2}$	119
10^{-3}	132
no antiserum	164 *

† Plaque size was smaller than normal.

* Numerous secondary plaques were obvious.

It was found that medium without antiserum contained many secondary plaques. Dilution of the stock antiserum preparation by 10^{-2} produced an overlay which had an inhibitory effect on plaque development. Plaque size increased as the antiserum concentration decreased. Optimum results were obtained with antiserum diluted 10^{-3} when there appeared to be no formation of secondary plaques but when the primary plaques developed to a size just short of that obtained with a Methocel overlay.

Since the adsorption period with the antiserum overlay was extended these results cannot be compared directly with results obtained using the two other overlays. However, an approximate comparison of the sensitivity of these methods was made and the results shown in Table 4 were obtained.

Table 4. Comparison of three types of overlay in vaccinia plaque titrations.

Overlay medium	Inoculum volume	Number of plaques per culture	Number of plaques per ml
Liquid medium	3 ml	164	55
Antiserum at 10^{-3}	3 ml	132	44
Agar at 1.2 per cent.	1 ml	41	41
Methocel at 0.75 per cent.	1 ml	48	48

Results obtained by all three methods were comparable. Either agar, Methocel, or homologous antiserum prevented formation of secondary plaques as far as could be judged by plaque counts and plaque size. Methocel was selected for use in standard overlays because this was technically easier to handle than agar, more readily available, and more constant in composition than homologous antiserum. As stated earlier it was essential that cultures being incubated with Methocel overlays should remain undisturbed throughout incubation.

From these preliminary experiments the standard plaque titration technique for poxviruses was selected. Monolayers of RK_{13} cells in 6 cm petri dishes were prepared, drained and inoculated with 1 ml of virus diluted in serum-free galactose medium. Adsorption was

for 3 hours at 37°C with occasional gentle rocking. Overlay containing 0.75 per cent. Methocel was then added, 10 ml per culture, without prior removal of residual inoculum fluid. Incubation was for 44 or 68 hours, depending on the virus, at 37°C in an atmosphere enriched with 5 per cent. carbon dioxide. To examine for plaques, cultures were drained and stained with 0.1 per cent. methyl violet in saline.

SPECIFIC RECEPTORS FOR POXVIRUSES

1. General Stability of Vaccinia Virus

Experiments were carried out to investigate the hypothesis that there existed on the surfaces of susceptible cells, specific receptor sites for poxviruses. The test method used was basically that described by Quersin-Thiry (1961) and Holland and McLaren (1961) in the investigation of specific receptors for poxviruses, enteroviruses and some others. Virus was mixed with a suspension of cellular homogenate and incubated thus for a stated length of time. The preparation was then assayed for residual virus activity. A decrease in virus titre was taken as indicative of attachment of virus to susceptible areas on the cellular debris with the consequent inability to attach to and initiate infection in a viable cell in the following titration. By this method and further confirmatory experiments the existence of specific receptor sites has been conclusively shown for enteroviruses by Holland and McLaren (1959), Holland et al. (1959) and McLaren et al. (1960).

Before starting work on the possible inactivation of vaccinia virus by homogenates of susceptible cells, some experiments were carried out to assess the general stability of vaccinia to suspension in various diluents, to heat inactivation and to the effects of ultra-sonic

vibration. Unless stated otherwise, the Lister strain of vaccinia was used.

Stock virus was suspended in a range of diluents and incubated for 1 hour at 37°C. Aliquots were then removed, diluted in serum-free galactose medium (SF gal) and titrated for residual infective virus by the plaque technique. The diluents tested are indicated in Table 5. These included suspending the virus in a standard preparation of RK_{13} homogenate. The effect of adding 10 per cent. calf serum to the galactose medium (10 per cent. CS gal) was tested. The control preparation was titrated immediately the stock virus was thawed and diluted: this sample was not suspended at 37°C prior to titration. The results obtained are shown in Table 5.

It was seen from Table 5 that the Lister strain of vaccinia had maximum stability under the conditions of this test when incubated with a homogenate of RK_{13} cells. The virus was remarkably stable in distilled water but was inactivated to some extent in saline. Virus stability was not increased by adding calf serum to the galactose medium. Since the virus was moderately stable in this medium and the pH remained stable during ordinary incubation, serum-free galactose medium was selected as routine virus diluent.

The stability of vaccinia virus to continued

Table 5. Stability of vaccinia virus when incubated for 1 hour at 37°C in a range of diluents.

Suspending fluid	Virus survival as pl.f.u. per standard volume
Control*	431
Saline	241
10 per cent. CS gal	305
SF gal	308
Hanks BSS	306
Distilled water	370
RK_{13} homogenate	495

* The control was a sample titrated immediately upon thawing the stock virus suspension.

incubation at 37°C in serum-free galactose medium was investigated. Results obtained are shown in Table 6.

Table 6. Stability of vaccinia virus to incubation at 37°C in serum-free galactose medium.

Duration of incubation at 37°C (in hours)	Virus survival as pl.f.u. per standard volume
0	238
½	178
1	186
2	123
3	115

It is shown in Table 6 that there is some loss of virus through incubation at 37°C prior to titration. The loss of virus increases as the incubation period increases. Approximately one quarter of the virus is lost after incubation for one hour and one half lost after 3 hours.

The effect of ultra-sonic vibration on the stability of vaccinia virus was investigated since this method has been used to disaggregate clumps of poxviruses and produce a mono-dispersed inoculum. The effect of ultra-sonication was tested on concentrated virus preparations suspended in serum-free galactose medium and in

galactose medium supplemented with 1 or 10 per cent. calf serum to determine whether extra protein stabilizes the virus. The duration of the ultra-sonic treatment and the results obtained are shown in Table 7.

Table 7. The effect of ultra-sonic vibration on the infectivity of vaccinia virus.

Duration of ultra-sonic treatment (in minutes)	Percentage survival of virus when suspended in galactose medium containing:				
	no calf serum A	B	C	1 per cent. calf serum	10 per cent. calf serum
10	26	22	95	47	62
5	28	36	-	-	-
2	29	29	90	53	40
1	26	24	45	-	48

A, B and C are repeat experiments.

- , no test carried out.

The collected results in Table 7 indicate a variable degree of inactivation but in general it appears that most inactivation occurs within the first 1 or 2 minutes leaving a resistant fraction of virus. The presence or absence of calf serum appears to have little consistent effect.

In dealing with a probe type of ultra-sonic disintegrator it is difficult to achieve identical experimental conditions. The probe does heat up during use

and may do so to a variable extent depending on how long the machine has been running, the initial temperature of the probe and other factors. Moreover, it is necessary to tune the equipment to the required frequency for each sample. The time taken to reach the required frequency and even the absolute value reached varies from sample to sample. These factors may contribute in part to the variation in results shown in Table 7.

2. The Effect of Incubating Some Viruses with Tissue Cell Homogenates

a) The Lister strain of vaccinia virus

To investigate the presence of specific receptors for the Lister strain of vaccinia on susceptible cells, preparations of virus were mixed with cell homogenate, incubated for either 1 hour at 37°C or 2 hours at 25°C, diluted and titrated for residual virus activity. Homogenates of HeLa cells, of RK_{13} cells and of Glax. RK_{13} cells were tested. The mixtures were incubated with continual shaking and the reaction stopped by dilution. Residual virus was titrated on HeLa cells, on RK_{13} cells and on Glax. RK_{13} cells.

Out of 24 experiments inactivation of virus, about 75 per cent., was recorded on three occasions, slight inactivation, about 15 per cent., was recorded on four occasions and in the remaining 17 experiments there was a distinct protective effect observed as compared with control samples incubated in serum-free galactose medium. The controls for all these experiments were samples which were incubated in galactose medium under conditions identical to those for the virus-homogenate mixtures. The results of these experiments are gathered together in Table 8, where they are expressed as percentage survival of virus after incubation in cellular homogenate. Virus survival of 100 per cent. would indicate identical survival in galactose medium

Table 8. Survival of vaccinia virus (Lister strain) after incubation for 1 hour at 37°C in homogenates of susceptible cells.

Type of homogenate	Percentage virus survival
HeLa	11
HeLa	19
HeLa	25
HeLa	79
HeLa	81
HeLa	112
HeLa	118
HeLa	132
HeLa	150
HeLa	157
HeLa	192
HeLa	197
HeLa	202
HeLa	210
HeLa	457
RK_{13}	81
RK_{13}	86
RK_{13}	123
RK_{13}	155
RK_{13}	158
RK_{13}	173
RK_{13}	191
RK_{13}	192
RK_{13}	355
Glax. RK_{13}	314
Glax. RK_{13}	593

and in homogenate. Less than 100 per cent. indicates an inactivating effect of the homogenate. Greater than 100 per cent. indicates a protective effect exerted by the homogenate.

Percentage virus survival =

$$\frac{\text{Number of virus particles surviving incubation in homogenate}}{\text{Number of virus particles surviving incubation in SF gal}} \times 100$$

The first three results given in Table 8 showing inactivation of vaccinia virus by HeLa cell homogenates were the first results obtained. While this does not invalidate them it must be mentioned that as the experiments progressed technique improved. It seems from the main bulk of results that cellular homogenate exerts no inactivating effect on vaccinia virus. On the contrary, increased virus yield was obtained after incubation in homogenate. The extent of the increase varied widely. There was no relationship found between the type of homogenate used and the type of cell monolayers used in subsequent titration. Pretreatment of homogenate tended to cause a reduction in the 'protective' effect of the homogenate, but the extent of this was not constant. It varied from 20-150 per cent. on the scale given in Table 8.

b) <u>The Evans strain of vaccinia virus and cowpox virus</u>

These results were unexpected since Quersin-Thiry (1961)

Table 9(a). Survival of Evans vaccinia after incubation for 1 hour at 37°C in homogenates of susceptible cells.

Type of homogenate	Percentage survival of Evans vaccinia
HeLa	1200
HeLa	140
HeLa	126
RK_{13}	1236
RK_{13}	105
RK_{13}	134
Glax. RK_{13}	1536
Glax. RK_{13}	144
Glax. RK_{13}	125

Table 9(b). Survival of cowpox virus after incubation for 1 hour at 37°C in homogenates of susceptible cells.

Type of homogenate	Percentage survival of cowpox virus
HEp-2	104
RK_{13}	142
Glax. RK_{13}	139

had reported the inactivation of vaccinia virus by homogenates of chick embryo fibroblasts and of HeLa cells. It was possible that the difference in results was due to use of a different strain of vaccinia. To investigate this, the effect of cellular homogenates on a genetically distinct vaccinia strain and on the closely related cowpox virus was measured. Experiments were carried out as for the Lister strain of vaccinia virus using the same homogenate stocks with the addition of a HEp-2 cell homogenate in one experiment. Surviving virus was titrated on RK_{13} monolayers. The results obtained, expressed as percentage virus survival, are shown in Tables 9 (a) and (b).

As for the Lister strain of vaccinia a protective effect was observed when Evans vaccinia or KP cowpox virus was incubated with homogenates of susceptible cells. The extent of the increase in titre after incubation of virus in cell homogenates varied widely and followed no obvious pattern.

c) Poliovirus type 1

Inactivation of poliovirus by homogenates of susceptible cells was demonstrated conclusively by Holland and his co-workers. Some tests were carried out therefore to see if inactivation of poliovirus by the homogenates prepared for vaccinia experiments could be demonstrated. Poliovirus type 1 was used. This

Table 10. Survival of poliovirus type 1 after incubation for 1 hour at 37°C in homogenates of susceptible and of insusceptible cells.

Type of homogenate	Survival of virus expressed as a 50 per cent. tissue culture infective dose		
	A	B	C
Control	$10^{-5.6}$	$10^{-4.8}$	$10^{-4.6}$
RK_{13}	-	$10^{-4.7}$	$10^{-4.5}$
Glax. RK_{13}	$10^{-5.7}$	-	-
HeLa	-	$10^{-4.0}$	$10^{-4.0}$
HEp-2	$10^{-4.7}$	-	-

A, B and C are repeat experiments.

- , no test carried out.

Control = sample incubated in serum-free galactose medium.

virus was able to grow in HeLa and in HEp-2 cells but not in RK_{13} or in Glax.RK_{13} cells. The inactivation experiments followed the same pattern as those preceding but in all cases the inactivation period was for 2 hours at 25°C. Control samples were incubated in serum-free galactose medium. Virus was titrated in tube cultures of HEp-2 cells or primary monkey kidney cells and the 50 per cent. tissue culture infective dose, TCD_{50}, was calculated according to the method of Reed and Meunch (1938). Results obtained are shown in Table 10.

Results shown in Table 10 indicate that by this procedure the infectivity of the poliovirus preparation was reduced by almost 90 per cent. by homogenates of HeLa or HEp-2 cells but unaffected by homogenates of RK_{13} or Glax.RK_{13} cells. These results are in agreement with those of Holland showing inactivation of poliovirus by homogenates of susceptible cells but not by homogenates of insusceptible cells. The fact that inactivation was demonstrated in this case lends support to the negative results obtained with vaccinia and cowpox viruses. The experimental model appeared to be satisfactory.

d) Adsorption of virus to viable cells

It was suggested from the work of Holland and McLaren (1959) that inactivation of poliovirus by the homogenates of susceptible cells was a result of contact

of virus with a specific receptor area causing the initial stages of virus eclipse. In order to demonstrate, with vaccinia virus, that experimental conditions for testing homogenates were suitable for the initiation of the virus eclipse phase, tests were carried out in which cell homogenates were replaced by viable cells. Virus was mixed with suspensions of viable cells for 1 hour at 37°C. Control samples of virus were mixed for 1 hour at 37°C in the serum-free galactose medium. All specimens were subjected to ultra-sonic treatment for 2 minutes and then titrated on RK_{13} monolayers. Results obtained are shown in Table 11.

Table 11. Survival of vaccinia virus after incubation for 1 hour at 37°C in viable cells followed by ultra-sonic disruption of cells.

Type of cell suspension	Virus survival as pl.f.u. per unit volume
Control	202
HeLa	114
Glax. RK_{13}	161

It was shown in earlier experiments that ultra-sonic treatment for 2 minutes causes some inactivation of virus but it was hoped the degree of inactivation was approximately constant. This will be discussed later as will loss of virus in the control sample through

adsorption to glass, since both factors probably contribute to lower the virus count in the control sample. However, it was shown, see Table 11, that lower virus yields were obtained after incubation of virus with viable cells and subsequent ultra-sonic disruption of cells indicating that under the conditions of test virus was able to adsorb the cells and enter eclipse. It appeared that virus was eclipsed more quickly in HeLa cells than in Glax. RK_{13}.

The following experiment was carried out to confirm the preceding experiment without involving the complication of ultra-sonic treatment and also to demonstrate loss of virus during incubation in the absence of cells. As before virus was mixed with HeLa cell suspensions, Glax. RK_{13} suspensions or with serum-free galactose medium and incubated with agitation for 1 hour at 37°C. Samples were withdrawn and titrated. The remaining virus suspensions were centrifuged at 750 g to deposit cells and thus any cell-associated or eclipsed virus. Aliquots were withdrawn from the supernate and titrated. Results obtained are shown in Table 12.

Table 12. Incubation of vaccinia virus for 1 hour at 37°C in viable cells with subsequent titration of the suspension and of the supernate after centrifugation.

Type of cell suspension	Virus survival as pl.f.u. per unit volume in:	
	whole suspension	supernate
Control	140	142
HeLa	266	196
Glax. RK_{13}	257	209

It can be seen from Table 12 that there was considerable virus loss in the sample incubated in serum-free galactose medium as compared with those incubated with either HeLa or Glax. RK_{13} cells. In this experiment virus attached to a viable cell which is subsequently plated out on a susceptible cell monolayer will give rise to a plaque so that the counts shown in the first column represent total virus both adsorbed and unadsorbed. Figures in the second column represent unattached virus which remained after the cell-associated virus was removed by deposition of cells. Table 12 shows that under the conditions of the experiment some virus is able to attach to cells but there is no significant difference between the results from HeLa cells and those from Glax. RK_{13} cells.

3. An Investigation of the Protection of Vaccinia Virus by Tissue Cell Homogenates

Preceding experiments have confirmed that poliovirus was inactivated by incubation in homogenates of susceptible cells. However, it was found that neither vaccinia nor cowpox virus were inactivated by cell homogenates under conditions of test which were shown to be suitable for adsorption of virus and entry into eclipse in viable cells. Out of a total of 36 experiments, distinct virus inactivation by homogenates was observed in only 3 cases, little effect on the suspended virus was observed in 4 cases and in the remaining 29 a marked increase in virus titre was observed as compared with controls incubated in a buffered medium. An attempt was made to elucidate the mode of action of cellular homogenates in either increasing the virus titre or preventing it decreasing. Experiments were designed to investigate several explanations.

a) The cellular location of the protective material

Firstly, cell homogenates were fractionated by differential centrifugation according to the method of Holland and McLaren (1961). The effect of each fraction on virus stability was measured in an attempt to determine the location in the cell of the protective material. The experimental model was identical to that used in complete homogenate tests and residual virus was titrated

on RK_{13} monolayers. The results obtained are shown in Table 13 and are expressed as percentage virus survival based on the 37°C control as base line. The control samples were, virus incubated in the serum-free galactose medium for 1 hour at 37°C and 4°C with agitation as for other samples.

Results shown in Table 13 and in repeat tests indicated that a greater 'protective' effect on virus was found with each of the individual fractions than with the complete homogenate. In all cases the homogenate and cell fractions were prepared and used at a final concentration equivalent to that from 10^7 complete cells per ml. From a comparison of the 37°C and 4°C control samples, and from many other similar readings, there appeared to be some inactivation of virus through heating for 1 hour at 37°C. The increase in titre through the action of cellular homogenates or fractions was consistently greater, although to a variable extent, than the increase found by keeping a control for 1 hour at 4°C. In describing these results it is difficult to know when to use the terms increase and decrease since these depend entirely on the base line chosen for calculations. As indicated earlier, virus titres greater than that obtained after incubation for 1 hour at 37°C in serum-free galactose medium will be described as increases, less than this, decreases.

Table 13. Survival of vaccinia virus after incubation for 1 hour at 37°C in various fractions of susceptible cells.

Fraction of RK_{13} cell homogenate in suspension	Percentage virus survival; as pl.f.u. per unit volume
Nuclear	136
Mitochondrial	160
Microsomal	175
Post-microsomal	188
Cell sap	173
Complete RK_{13} homogenate	119
Control at 37°C	100
Control at 4°C	110

b) The nature of the protective material

Since the 'protective' effect of cell debris on virus could not be located in any fraction, the next attempt to elucidate the problem was made by subjecting homogenates to various physical and chemical tests in the hope that this might give some indication of the nature of the 'protective' material. Homogenates of RK_{13} cells were treated as follows:

i. heated for 30 minutes at 60°C;

ii. ultra-sonicated for 5 minutes;

iii. ultra-sonicated for 10 minutes;

iv. incubated for 1 hour at 37°C in 0.5 per cent. trypsin, centrifuged to deposit the homogenate, and re-treated with the trypsin preparation;

v. a trypsin control in which homogenate was replaced by an equivalent volume of serum-free galactose medium was included;

vi. incubated for 2 hours at 37°C in 0.3 per cent. lipase;

vii. a lipase control in which homogenate was replaced by an equivalent volume of serum-free galactose medium was included;

viii. incubated for 1 hour at 37°C in 10^{-3} M sodium metaperiodate after which glycerol was added to the same molar concentration;

ix. as for viii replacing the homogenate by serum-free galactose medium.

Table 14. Survival of vaccinia virus after incubation for 1 hour at 37°C in homogenates of susceptible cells which had been subjected to various physical and chemical treatments.

Pre-treatment of RK_{13} cell homogenate	Percentage survival of virus after homogenate treatment; as pl.f.u. per unit volume
normal homogenate	143
heated for 30 mins at 60°C	171
u-s for 5 mins	136
u-s for 10 mins	150
0.5 per cent. trypsin	257
trypsin-virus control	21
0.3 per cent. lipase	2
lipase-virus control	14
10^{-3} M periodate	100
periodate-virus control	43
no homogenate control	100

u-s = ultra-sonicated

Preparations which had been treated in this way were mixed with virus, incubated with mixing for 1 hour at 37°C, diluted and titrated. Controls included were a homogenate preparation which had had no pretreatment and another in which homogenate was replaced by serum-free galactose medium. The results are shown in Table 14 where virus survival is expressed as a percentage calculated from the serum-free galactose medium control as base line.

The percentage virus survival in normal homogenate was 143 per cent. An increase in virus survival was obtained when homogenate had been treated prior to the test by heating, ultra-sonication, or the action of trypsin. Virus survival was reduced in homogenate preparations pretreated with lipase or metaperiodate. However, these results must be interpreted with care since trypsin, lipase and metaperiodate-glycerol treatment of virus cause considerable viral inactivation.

c) <u>The mode of action of the protective material</u>

Some factors which may have accounted for the increase in virus titre, found when virus was incubated in the presence of cell homogenates, are listed below. It was possible that the increase was due to prevention of viral aggregation by homogenates; this will be considered in the discussion. Previous work suggested, see e.g. Table 13, that the homogenate may exert a

protective effect against heat inactivation. Another possibility considered was that homogenate somehow prevented loss of virus through adsorption on to glass. The two latter possibilities were investigated further.

A measure of heat inactivation was made by carrying samples of virus through parallel procedures, one at 37°C and one at 4°C. Loss of virus by adsorption on to glass was investigated by increasing the glass surface area, either by introduction of ballotini beads or by shaking the reaction vessel. As before experiments were carried out in 25 ml pyrex conical flasks with cotton-wool stoppers. Total reaction volume per test was 1 ml and when ballotini beads were used 0.5 gm wt was added to each sample. Tests at 37°C were carried out in water-baths, either stationary or with mechanical agitation at a constant speed. Tests at 4°C were carried out in a refrigerator using pre-cooled flasks and solutions. The duration of each test was 1 hour and virus titrations were on RK_{13} monolayers. The results obtained in such a test are shown in Table 15.

When mechanical agitation was used in these tests it was gentle and considered unlikely to damage the virus particles. Results shown in Table 15 demonstrate clearly a loss of virus as the glass surface area increased by the introduction of glass beads. In three out of the four pairs there was further virus loss by

Table 15. The effect of glass surface area on the loss of vaccinia virus from a suspending fluid.

Treatment of sample	Number of pl.f.u. per unit volume of sample
37°C - beads - agitated	8
37°C - beads - stationary	59
37°C - agitated	126
37°C - stationary	118
4°C - beads - agitated	60
4°C - beads - stationary	66
4°C - agitated	104
4°C - stationary	130

agitation of the cultures during the reaction period. Only the first of the four pairs showed significant heat inactivation of virus. From these results it was apparent that adsorption of virus to glass surfaces accounted for a large proportion of virus loss in control samples in all preceding experiments. Both there and here the virus suspending fluid was the serum-free galactose medium. Heat inactivation of virus may have been a contributing factor, but to a much lesser extent.

It then remained to demonstrate that cell homogenates could prevent loss of virus by adsorption to glass. A similar type of experiment to the above was carried out. Glass surface area was increased by the addition of ballotini beads and the effect of incorporation of cell homogenates in such test samples was measured. The conditions of test and results obtained are shown in Table 16.

Results shown in Table 16 indicate that the progressive loss of virus with increasing surface area of reaction vessel was overcome by the presence of tissue cell homogenate. The effect of homogenate on the 4°C stationary samples was less than anticipated, but this may well have been due to inability of homogenates to reach virus in the absence of gentle shaking.

A control titration was carried out with an

Table 16. The protective effect of tissue cell homogenate against loss of vaccinia virus through adsorption on to glass.

Treatment of sample	Virus survival as pl.f.u. per unit volume
37°C - beads - agitated	8
37°C - beads - agitated - homogenate	330
37°C - agitated	65
37°C - agitated - homogenate	375
4°C - beads - stationary	45
4°C - beads - stationary - homogenate	285
4°C - stationary	110
4°C - stationary - homogenate	152
Sample titrated immediately upon thawing stock virus	340

aliquot of stock virus obtained immediately after thawing and without any pre-incubation at 37°C or 4°C.

PART I

Interaction between Cellular Homogenates and Vaccinia Virus

DISCUSSION

The growth of vaccinia virus in RK_{13} cells was compared with that in HeLa cells. The former cell line was selected as the titration system because it was more sensitive to infection with vaccinia virus and moreover, viral lesions developed faster in this than in the HeLa line. Similar findings were obtained (unpublished results) when the growth of rabbit-pox virus in RK_{13} and in the ERK line of HeLa cells was compared. In this case it was found that the increased viral growth rate was not due to increased rates of adsorption, penetration, or entry into eclipse, but probably to differences in the intracellular replication processes. Allison and Valentine (1960a) also found that the rates of adsorption of vaccinia were of the same order in cells in which the virus multiplied readily and those in which it did not. The RK_{13} cell line was therefore considered the more suitable for these poxvirus studies.

A general investigation was carried out on the stability of vaccinia virus to various forms of physical and chemical treatment. In particular, the effect of incubating virus in homogenates of susceptible cells was studied. It had been found by Quersin-Thiry (1961) that such conditions resulted in a 90 per cent. inactivation of virus when tests were carried out in pyrex glassware. These results were masked when the tests were carried out in ordinary glassware due to the

massive loss of virus by adsorption to glass. The work described here therefore, was carried out entirely in pyrex glass vessels. Inactivation of western equine encephalomyelitis virus and of Newcastle disease virus was also demonstrated and in neither case was the type of glassware used so important as in the case of vaccinia.

These findings were analogous to the inactivation of poliovirus and certain other enteroviruses by homogenates of susceptible cells found by Holland and McLaren (1959) and McLaren et al. (1960). Holland and his co-workers demonstrated that such inactivation was due to combination of virus with specific enterovirus receptor areas which exist on the surfaces of susceptible but not of insusceptible cells. Such attachment led to irreversible eclipse of infectivity on the intact cell and to a reversible eclipse when the virus combined with receptor material from disrupted cells (Holland, 1962).

No inactivation of vaccinia virus by homogenates could be demonstrated. On the contrary, homogenates of all cells tested exerted a protective effect on the virus. To confirm that the experimental model was not at fault, and in particular to confirm that the active material in the homogenate had not been destroyed by the method of preparation, the effect on poliovirus type 1 was tested. The expected inactivation of virus

was found with homogenates of susceptible cells while no inactivation was observed with homogenates prepared from insusceptible cells (see Table 10).

Since the process of inactivation is envisaged as being due to combination of virus with that area of the cell to which the virus normally adsorbs prior to penetration and eclipse, experiments were carried out to confirm that conditions were suitable for normal adsorption of virus to viable cells (see Table 12). Virus adsorption to such cells occurred under the conditions of test. These results suggested that while fragments of cells, the postulated viral receptors, may have attached to virus they had no detectable adverse effect on viral infectivity. Higher virus titres were consistently demonstrated after incubation in the presence of cell homogenates as compared with incubation in growth medium.

An attempt was made to determine which fraction of the homogenate was responsible for this increase in virus titre. Survival of virus after incubation with different cell fractions was measured (see Table 13). It was found that the protective effect of each of the cell fractions, namely, nuclear, mitochondrial, microsomal, post-microsomal and even cell sap was greater than the effect of the whole homogenate. Holland and McLaren (1961) had carried out similar experiments to determine the location of the enterovirus inactivating

fraction of susceptible cell homogenates. They found greater activity in the microsomal fraction than in the complete homogenate and less activity in the mitochondrial, nuclear, post-microsomal and cell sap fractions, decreasing in that order. The high protective activity of all cell fractions, including the cell sap fraction, reported here with vaccinia virus, indicates a much more non-specific type of reaction.

The stability of the protective material in the homogenate to a series of physical and chemical reactions was investigated (see Table 14). The effect of the homogenates was not removed by heating for 30 minutes at 60°C or by ultra-sonic treatment. The proteolytic enzyme trypsin, had an interesting effect. Trypsin treatment of the virus alone caused considerable reduction in viral infectivity. When the homogenate was treated with trypsin prior to reaction with the virus, a significant increase in the protective effect of the homogenate resulted. This and the other tests shown in Table 14 were carried out exactly as described by Quersin-Thiry and Nihoul (1961). These workers found that the action of 0.5 per cent. trypsin on vaccinia virus alone resulted in an increase in virus titre which they suggested was due to the dissociation of viral aggregates. They, therefore, did not examine the effect of trypsin on homogenates. The results from tests carried out here showed quite the reverse

trend. These might be explained by the assumption that while 0.5 per cent. trypsin inactivates the virus (see Table 14) the amount of enzyme remaining after trypsin-homogenate interaction was insufficient to inactivate the virus but sufficient to dissociate viral aggregates. Alternatively, trypsin may act by splitting cellular debris into smaller but still active fragments and thus increase the virus protective effect. It would suggest, however, that that part of the cellular material responsible for viral protection does not have a highly specific protein configuration. The effect of lipase could not be measured, since this enzyme at concentrations of 0.8 per cent. and 0.3 per cent. had such a marked antiviral effect on the virus alone. This also is not in agreement with the results of Quersin-Thiry and Nihoul (1961), who found that lipase at 1 per cent. concentration had no adverse effect on vaccinia. Also in contrast to these workers, it was found that metaperiodate had an inactivating effect on vaccinia. Considerable virus survived, however, after incubation with homogenate which had been pretreated with metaperiodate. Although it is impossible to interpret these results with certainty because of the antiviral action of the chemical, this may indicate that the protective effect of the homogenate is little affected by periodate and, therefore, not principally carbohydrate in nature. The difference between these results and those obtained by Quersin-Thiry may be

partly explained by a difference in the state of the virus preparation under test. The preparation used in these experiments had been purified by differential centrifugation and was reasonably free from cellular debris. That used by Quersin-Thiry does not appear to have been so treated. The activity of enzymes and other chemicals may have been masked by the presence of cellular debris in the unpurified preparation.

These results, considered together, indicate that the protective material in homogenate is dispersed throughout the cell; is resistant to mild heat inactivation; is resistant to physical disruption by ultrasonic treatment; and probably does not consist of specific protein or carbohydrate configurations. The mechanism of protection appears to be a non-specific reaction.

Some experiments were then designed to determine how the homogenate exerted its protective effect. Several possibilities were considered. It was possible that the homogenate acted by combining with virus in some way which reduced heat inactivation. Alternatively, it may have been effective in reducing aggregation of virus, or even by dissociating aggregates already formed. The final possibility considered was that the active material exerted its effect by preventing the loss of virus through adsorption to glass.

Incubation of virus under various conditions was

carried out in parallel at 37°C and at 4°C and the survival of virus at the two temperatures compared. Results in Table 15 indicate that there might have been slightly greater inactivation at 37°C than at 4°C but this was marginal. It is unlikely that the significant difference in virus survival found in the presence and absence of cell homogenate could be fully accounted for by stabilization against heat inactivation.

The increase in virus titre observed after homogenate treatment may have been due to disaggregation of viral clumps by enzymes present in the preparation of disrupted cells. Several pieces of evidence served to eliminate this possibility. The activity of a homogenate was not removed by heating for 30 minutes at 60°C, conditions which would inactivate normal cellular enzymes (see Table 14). Moreover, maximum activity was observed in the heaviest cell fractions, e.g. the nuclear and the mitochondrial fractions, which came down first in the cycle of differential centrifugation and were probably reasonably free from cellular enzymes. That fragments of cellular material combined with virus particles preventing aggregation, and thus the effective loss of virus, was more difficult to investigate. Ultra-sonic vibration as a means of dissociating aggregates was of limited use, since even treatment for one minute inactivated a large proportion of virus (see Table 7).

McLaren (personal communication) had carried out some studies in an effort to detect specific binding of vaccinia virus by homogenates of HeLa and other cells and was also unable to detect any significant interaction. He found a similar increase in titre after incubation in homogenate as compared with virus controls and ascribed this to de-clumping of virus. He did not distinguish between disaggregation of performed clumps and prevention of viral aggregation. From the results found here the former appears unlikely, but the latter cannot be eliminated. However, in the light of later glass adsorption studies, it probably was not of major importance.

What appeared to be of primary importance was the action of homogenate in preventing loss of virus through adsorption to glass. It was found (see Table 15) that as the surface area of the glass reaction vessel increased, all other factors remaining constant, the survival of virus decreased. Moreover, when the homogenate was added to the virus suspension and incubation at 37°C continued (see Table 16), virus survival was the same regardless of glass surface area. The virus titre obtained after incubation of virus with homogenate was the same as the titre of stock virus, indicating 100 per cent. survival. Preliminary studies have suggested that virus loss through adsorption to glass was decreased when silicone-coated

glass was used.

Valentine and Allison (1959) and Allison and Valentine (1960a, b) have compared the adsorption of virus particles to biological and non-biological surfaces. They found (1959) that the rate of attachment to glass agreed closely with the results expected from Brownian theory, namely, that each collision resulted in attachment. When vaccinia virus was mixed with HeLa cells in suspension they found that the rate of adsorption was only one third of that expected or that only one third of the collisions resulted in virus attachment. Thus, the efficiency of adsorption to cells was less than that to glass. Considerable loss of virus by adsorption to glass must be expected when virus suspensions are incubated in glass vessels. This was found throughout these experiments. Such loss of virus was diminished by the addition of cellular homogenate to the suspending fluid. Although the efficiency of adsorption to the cellular material is less than that to glass, when homogenate is in suspension, there is a smaller distance over which the virus has to pass to reach homogenate than to reach the glass walls of the vessel. It was found that when samples were incubated at $4^{\circ}C$ without agitation (Table 16), the protective effect of homogenate was much reduced, probably due to reduced effective surface area and reduced availability of the homogenate.

The cellular material may act by combining with virus particles and so preventing the attachment to glass. Alternatively, the cellular material may become adsorbed to the glass conferring on it hydrophilic properties and so hinder virus attachment as suggested by Valentine and Allison (1959) in a consideration of a similar protective effect of protein.

The question remains whether or not the absence of inactivation by binding with cell homogenates indicates the absence of specific receptors for vaccinia virus on the surfaces of susceptible cells. Inactivation of virus by cell receptors requires either the initiation of viral eclipse or else the formation of a complex that is unable subsequently to attach to a viable cell. It is possible that virus-receptor binding could occur without either of these results ensuing. In particular, with regard to viral eclipse, Joklik (1962a) has presented evidence for the action of a series of enzymes in the initiation of poxvirus eclipse and it is possible that this degradation mechanism was rendered totally ineffective when the cells were homogenised by the method used here. The one condition, namely, that tissue cell homogenate can inactivate virus is insufficient to determine whether or not specific receptor areas for poxviruses do exist on cell surfaces.

The finding here that there is marked protection of vaccinia against adsorption to glass does not prove

that the attraction of cell debris for virus is greater than that of glass. As discussed the result may be due to coating of glass by homogenate. Moreover, Valentine and Allison (1959) have evidence to the contrary showing that the efficiency of vaccinia attachment to such non-biological surfaces as glass, gold and aluminium, is greater than to susceptible cells. They have further shown (1960b) from the temperature coefficient of attachment, the effect of ions and other properties, that the primary attachment of poxviruses to cells is electrostatic in nature.

It would appear from a consideration of these results and from the very wide host range of this virus that the initial attachment of vaccinia virus to the host cell is by some reaction of low specificity which is electrostatic in nature.

PART II

A Tissue Culture Model of a Latent Infection with Herpes Simplex Virus

INTRODUCTION

In a consideration of latency in viral infections the first major problem encountered is definition of terms. The ability of a virus to invade a cell and to remain hidden there for some variable interval, until later reactivated by a suitable stimulus, has been described in many ways. Such conditions have been called latent, masked, inapparent, sub-clinical and carrier states. Different terms have been used to make fine distinctions among the phenomena described and to this extent each may have been valid. However, it is difficult to separate such latent infections into types or groups until a more fundamental understanding of these processes is available. The situation was fast approaching where individual workers selected terms for their individual purposes and not according to any general pattern. To attempt to survey the subject at this stage without immense background knowledge resulted in total confusion. Andrewes (1958) appreciated this problem and brought some order to the field by a grouping of terms and by more stringent definition. This was essential before the problem of latency as a whole could be considered.

The attempt will be made here to approach the problem as though using a microscope and to move progressively from low magnification to high; that is to move from a general consideration of latency, through the situation in the whole animal, through the situation

at a cellular level, to the sub-cellular and even to the molecular level. However, the optical aberrations associated with magnifying devices must be borne in mind when later translating the findings of a molecular study to the intact animal. For this purpose latency in a viral infection will be considered as that state of equilibrium achieved between host and virus, when the infectivity of the virus is reversibly masked for a period longer than the normal eclipse phase of the host-virus system.

At the level of the whole animal, the term latency must cover the activity of those viruses that result in long lasting inapparent infections. In these the presence of the virus is often difficult to demonstrate but there is always the possibility that the infection may become activated and overt. Latent infections in man and animals fall into two groups. Firstly, there are those in which the infection is contracted by the foetus in utero and is transmitted from generation to generation by this means. Secondly, there are those infections which occur following a variable interval after birth and which may persist throughout life.

An example of an intra-uterine infection is lymphocytic choriomeningitis of mice (Traub, 1938). Animals carrying this virus never indicate the presence of infection by clinical signs but do show certain

distinctive pathological lesions which are the result of infection (Traub, 1936). This carrier state is due to a form of immunological tolerance exhibited by the host towards the virus. It is the result of the host's being infected with the virus before it is capable of immunological response.

There are a number of examples of latent infection in animals in which the virus is initially encountered early in life. One virus acting in this way is the pneumonia virus of mice (Horsfall and Hahn, 1940). This agent may persist in the lungs of mice for an indefinite period but is not detectable until it causes a spontaneous viral pneumonia or until it is uncovered by serial passage of lung tissue through susceptible mice. Virus III, which is a type of herpesvirus affecting some stocks of rabbits, acts in a similar way (Rivers and Tillett, 1924). It has also been demonstrated that vaccinia virus can persist in rabbits which have recovered from infection (Olitsky and Long, 1929). Another very important group of latent infections is the ornithoses of a variety of birds (Meyer, 1952) but these should be considered separately as these agents belong more properly with the bacteria and rickettsiae than with the viruses.

The one infection in man in which a latent stage has been clearly demonstrated is Herpes simplex. Infection with this virus is usually acquired in very

young children and then in most cases persists throughout life (Anderson and Hamilton, 1949). The initial infection in children may be accompanied by a primary disease, most frequently stomatitis, but this may range from a mild vesicular eruption to a fatal meningoencephalitis. After the primary infection there is a marked tendency to recurrence but subsequent attacks are usually less severe. Primary infection can occur in later life and herpetic lesions may appear on the cornea or on the genitals. In the former instance recurrent attacks often lead to progressive scarring of the cornea and ultimate blindness. Presence of antibody in the blood does not prevent recurrences.

Factors involved in herpetic exacerbation are little understood. A variety of stimuli have been implicated such as fever, due to one or other of several causes, e.g. pneumococcal lobar pneumonia, malaria, influenza, T.A.B. vaccination; cold; sunlight; and emotional stress (Van Rooyen and Rhodes, 1948). These, however, have only been offered as explanations in the most general terms.

It has been suggested (Blank and Rake, 1955) that the relationship between chicken-pox and zoster is analogous to that of primary and recurrent Herpes simplex, namely, that zoster is a second clinical manifestation of infection with virus which may have remained latent in the tissues since an earlier

chicken-pox infection.

Certain adenovirus infections of man may possibly behave in a fashion similar to that of Herpes simplex. This is illustrated by the manner of discovery of this group of agents. Adenoviruses were first isolated when it was observed that surgically removed tonsils and adenoids grown in tissue culture underwent a characteristic unprovoked degeneration which Huebner et al. (1954) demonstrated to be the result of virus activity. This could be detected only after cultivation of the removed tissue cells and not by testing homogenates of the removed tissue on other cells. The explanation offered was that the tissue removed contained virus which persisted without obvious manifestations until environmental factors were altered by the initiation of in vitro growth.

These are some examples of latent infections caused by viruses. The phenomenon is not rare and many other instances could be cited. Latency is not confined to the animal kingdom. Plants too have their latent infections, e.g. paracrinkle virus in King Edward potatoes (Andrewes, 1958), or tobacco mosaic virus infection of the bean plant (Rappaport and Wu, 1963). These instances serve to illustrate the range and complexity of latent viral infections and to stress their importance. They state the problem; they do not offer a solution. The question remains as to what

controls this fine balance between host and virus so that neither grows out to the ultimate destruction of the other.

It is very difficult to attempt an explanation based on a study of the situation in the living animal. There are too many unknown variables to allow a detailed investigation. It is difficult to observe events, cytopathological or biochemical, in the infected cells even if such cells could be located. The obvious approach in an attempt to elucidate this problem is to set up a tissue culture model and in this to try to imitate the events occurring in the multicellular organism.

Many workers have been interested in this approach and several models have been prepared using different viruses and different cell systems.

Infection of cells at sub-optimal temperatures has been suggested as a cause of latency. Indeed Gohd (1958) produced cultures carrying latent influenza PR_8 virus for up to three weeks when held at 20°C, with the subsequent outgrowth of virus when the temperature was raised. Coleman and Jawetz (1961) maintained a latent infection in tissue culture with Herpes simplex virus for nine months by a similar method, and here too the virus was reactivated by elevation of temperature. In this case it should be noted, the cells were reported

as being morphologically abnormal, sometimes difficult to subculture and easily destroyed by trypsin. A latent infection of tobacco mosaic virus too, has been activated by elevation of temperature (Rappaport and Wu, 1963).

The implication of hormonal imbalance in stimulating a latent infection was suggested by Bittner (1942) in his work on oestrogen levels and initiation of mammary cancer in mice; also by Schmidt and Rasmussen (1960) who found that a latent herpesvirus infection in the rabbit could be activated by adrenalin. Conversely it may be possible that a similar mechanism operates in establishing a latent state. This line of approach does not appear to have been developed <u>in vitro</u>.

Another possibility exciting considerable interest recently is that latency may be the result of a dual infection either through the mediation of interferon or some other result of viral interference. Barski and Cornefert (1962) in the study of a herpesvirus latency in cells concomitantly infected with polyoma virus favoured the explanation that the latent state resulted from the action of polyoma-induced interferon. In a similar model Glasgow and Habel (1963) also tended to favour interferon as the active agent. They suggested that it acted either by protecting cells against growth of herpesvirus; or else that cells protected by polyoma-induced interferon, produced herpesvirus-induced

interferon more efficiently as a result of limited herpesvirus growth. However in this case, resistance was achieved only by the use of very low multiplicities of infection suggesting that secondary cycles of herpesvirus replication were suppressed. In an earlier paper Glasgow and Habel (1962) had discussed the effect of exogenous interferon on herpesvirus replication and the attainment of a latent state by this means. This might offer a fruitful approach to understanding the part interferon plays in latency. Rodriguez and Henle (1964), in the study of a persistent infection of Newcastle disease virus in tissue culture, also suggested that interferon was the operative mechanism, and that its production was stimulated by the action of non-infectious virus particles on cells. In this paper these workers offer a clear diagrammatic explanation of the process.

Many workers in this field have attempted to establish latent infections by infecting cells with virus in the presence of the homologous antiserum. Carrier cultures may result by this method for one or more reasons. Under the selection pressure of antiserum a virus of decreased virulence may emerge, as has been shown by Hinze and Walker (1961) in cells persistently infected with Herpes simplex virus. Alternatively cells of increased resistance may be selected as Fernandez (1960) found in her model of persistent herpesvirus infection. The possibility of

resistant cells emerging in this way had been suggested by Ackermann (1957) in a discussion of his carrier cultures of cells with poliovirus. Another way in which antiserum may act is by binding with the virus either outside or on the surface of the cell and thus restricting, but not completely eliminating, its activity. Ackermann (1958) has suggested that this may result in a greatly reduced rate of virus growth and that this is offset by continued growth of cells to give a balanced state. Alternatively antiserum may act by forming a reversible complex with the virus in which viral infectivity is masked until the complex is dissociated. The dissociation of a virus-antibody complex with subsequent recovery of viral infectivity has been demonstrated by Mandel (1961) working with poliovirus.

These systems, in which the effect of temperature, hormonal imbalance, dual infections and antisera are considered, are of value in an attempt to imitate natural phenomena under more rigorously controlled conditions. They throw more light on the generalizations hitherto produced, for example, that latent infections are activated by fever or by the activity of a secondary invader, and thus help to give an understanding of the problem at a cellular level.

Other workers have probed yet deeper and have tried to elucidate the relationship of virus and host in something approaching molecular terms. This is an effort to find the common pathways for viral latency achieved through a variety of mediators. It is in relating such findings to the condition of latent infection in the whole animal that the greatest of care must be taken.

At the sub-cellular level it seems reasonable to approach the problem along two fronts. A type of extracellular latency may be envisaged in which viral infectivity is neutralised by the formation of a reversible complex, as described above with reference to antiserum. Alternatively the latent state can be considered as the reversible suspension of the intracellular viral reproduction cycle.

The use of antiserum has already been mentioned and the difficulty of restricting its activity to one pathway discussed. The work to be described in this thesis principally concerns Herpes simplex virus. Since the object of all these studies has been to produce a simplified model of <u>in vivo</u> events, it is of interest here to consider the part played by the antibody response to herpetic infection in the animal host.

Firstly, antibody develops in man within a few days after onset of primary infection and then persists for an indefinite period. However it does not prevent

local recurrences of viral activity. Secondly, it has been shown (Wildy and Watson, 1962) that herpesvirus particles may occur in either a naked or an enveloped form. The naked particles were found to be agglutinable by antiviral serum. Those particles which were enveloped in one or two membranes derived from the cell in which they were produced, were agglutinable by anti-host cell serum and, it appeared, insensitive to the action of antiviral serum. Moreover it has been shown that both forms are infective (Watson et al., 1964). Thus it would seem that the latent state in vivo is unlikely to depend wholly on an antibody mechanism. Too many virus particles would always be protected from antiviral antibodies.

Another agent found to be inhibitory to several strains of Herpes simplex virus (Nahmias and Kibrick, 1964) and which is known to be present in various animal and human tissues is heparin (Riley, 1963). Heparin, which is a sulphated mucopolysaccharide, was the only one of several biological sulphated polymers tested found to inhibit Herpes simplex virus (Nahmias et al., 1964). Nahmias and Kibrick showed that heparin acted by forming a complex with the virus particle through the sulphate groups, thus preventing the primary electrostatic attachment of virus to cell. They showed in addition, that the complex formed was dissociable upon

dilution. This led them to the consideration that the activity of heparin may be an explanation for the ability of Herpes simplex virus to cause latent infections. The suggestion made was that, under certain stimuli, infective virus may become dissociated from the heparin complex and proliferate locally with further spread of infection being prevented by the presence of circulating antibodies.

The object of the second part of this study on latency in herpesvirus infection in tissue culture was to investigate the sensitivity of Roizman's MP strain of Herpes simplex virus (Roizman and Roane, 1963) to the action of heparin, and thereafter the possibility of producing a latent infection in tissue culture mediated by heparin.

The concept of latency as an intracellular phenomenon has been investigated in several ways. One possible method of inhibition of the intracellular viral replication cycle is by the use of synthetic antiviral agents. These compounds cover an extensive field of study. However they are of limited interest with regard to the elucidation of the basic mechanisms of latent viral infections. Structural analogues and other antiviral agents are not naturally occurring compounds. Many have been synthesised only recently

and therefore cannot be implicated as causative agents in latent infections. In developing a model of an *in vivo* phenomenon the object should be to achieve this by manipulation of conditions and materials which arise naturally and not too infrequently. The use of highly artificial conditions may serve to provide a system with some of the characteristics of a latent infection, but not to produce a model which imitates the natural processes and thereby may lead to a fuller understanding of intracellular events.

However, in passing, it must be mentioned that some very interesting work has been done on the psittacosis group by Pollard and Sharon (1963) using the folic acid antagonist aminopterin. Using this compound they induced a latency of up to 30 days duration. Within this period the psittacosis agent could be reactivated or 'recalled' by addition of folinic acid. It was also shown that while folic acid to some extent reversed the inhibitory effect of aminopterin on the cell and psittacosis agent, thymidine reversed the damage to the host cell only. Thus a difference was indicated in the metabolic pathways of the thymidine and deoxyribonucleic acid of the host cell and of the infecting agent. It is findings of this nature that should ultimately lead to a fuller understanding of the processes affecting intracellularly replicating parasites and give hope for a rational development of chemotherapeutic agents.

One of the most promising approaches to the problem of latency, to date, involves nutritional imbalance and conditions of partial starvation of cells. To develop this approach it is necessary to determine which nutrients are required most specifically for the growth of the infecting virus as opposed to the requirements of the cell. The hypothesis is that, if there is a high degree of specificity by the virus for one nutrient, the omission of that nutrient may result in the cessation of virus growth. Subsequent replacement of the missing component may initiate a resumption of virus growth. If this occurs a system of reversible suspension of the virus replication cycle has been achieved and this may be considered as a form of latency. It is not difficult to imagine such a situation in vivo. This is one way of removing a key molecule in the virus growth cycle, namely, by removing the essential building blocks; it avoids addition to the system of structural analogues or other synthetic antiviral agents.

This possibility has been investigated in considerable detail by Morgan and co-workers with regard to the psittacosis group. It is of value to consider this work here since it illustrates so well the method of studying latency from the aspect of altered cell nutrition. Morgan (1956) described a latent infection of psittacosis in chick embryo tissue

cells which he induced by infecting the cells after a period of starvation in a balanced salt solution. The infecting agent could be 'recalled' or resumption of replication could be initiated by addition of a beef embryo extract to the infected system at any period up to a total of 15 days suppression. Bader and Morgan (1961) attempted to identify the active fraction of the beef extract and found a range of amino acids, water soluble vitamins, inorganic salts and glucose were necessary to stimulate virus growth. They suggested from this work that protein of the psittacosis agent was synthesised from the cellular amino acid pool rather than from a breakdown of cellular proteins. Thus was emphasised the importance of the supply of nutrients to the cells on the ability of these cells to support growth of the infecting agent.

The effect of nutritional requirements for the growth of enteroviruses has been studied by several workers. It was reported by Eagle and Habel (1956) that only minute amounts of poliovirus were produced in media deficient in glucose and glutamine and that addition of both missing components to a medium resulted in a rapid proliferation of viral growth. Dubes (1956) obtained variable results on the omission of glutamine from the medium, but he found that in a medium lacking cystine the cytopathic effect of poliovirus in monkey kidney cells was arrested. Rappaport

(1956) stressed the importance of cysteine in the growth of poliovirus in monkey kidney cells. However, it appears that the absolute nutritional requirements for the growth of any particular virus depends on the cells which are infected. The requirement of poliovirus for cysteine varied according to the type of cell in which the virus was grown. Eagle and Habel (1956) demonstrated within one log of optimal yield of poliovirus in HeLa cells maintained in a medium containing only glucose, glutamine and salts. Tyndall and Ludwig (1960) showed near maximum production of poliovirus in HeLa and in rabbit kidney cells in a similar medium which again lacked cysteine. In the same paper Tyndall and Ludwig reported a requirement for cysteine of coxsackie B_3 and vaccinia virus growth in monkey heart cells. They showed further that increased starvation of cells led to the establishment of a latent coxsackie B_3 infection, the activation of which was cysteine dependant (Tyndall and Ludwig, 1963).

A study of the amino acid requirements for the growth of Herpes simplex virus in mouse L cells was made by Pelmont and Morgan (1959). They found that omission of certain amino acids from the growth medium resulted in a marked decrease in the growth of the virus while some others could be removed with little effect on virus proliferation. In studies on herpes-

virus-infected HeLa cells, Lewis and Scott (1962) found there was a marked requirement for both glucose and glutamine. The concentration of glutamine for optimal virus production was critical, either too high or too low a concentration of glutamine resulted in a reduced virus yield. They showed too that reduced virus growth in deficient media was attributable neither to decreased survival time of infectious particles or of cells, nor to decreased rate of viral adsorption or penetration. They concluded that the effect was most probably on intracellular viral replication. This is important in pursuing these studies in the hope of developing a model latent infection.

It is of interest to note that in a totally different system, that of measles virus in HEp-2 cells, Reissig et al. (1956) found that increased glutamine concentration in the medium suppressed the giant cell formation characteristic of this virus while a depletion of glutamine led to the formation of syncytial masses. These results were influenced by the calf serum content of the medium, most probably as a result of the glutamine content of the serum.

In a detailed analysis of the amino acid requirements for the growth of Herpes simplex virus in human cells, Tankersley (1964) showed the degree of requirement for each of the eleven amino acid components of Eagle's medium. The greatest specific requirements were for histidine and arginine and the least was for

lysine. Omission of histidine resulted in a curtailment of virus production but a continuance of viral cytopathic effect. At the other end of the scale, the omission of lysine was found to result in a slightly increased virus yield. Most interesting of all, Tankersley showed that omission of arginine resulted in a cessation of viral growth and of virus-induced cytopathic effect and that replacement of arginine in the medium resulted in a prompt and extensive resumption of the virus infective process.

Here is a system in which a slight alteration in the balance of nutrients controls the fate of the infecting virus. Several questions arise from these findings. What happens to the cell under conditions of arginine starvation? Since arginine is known to be an essential nutrient for the normal growth of cells in tissue culture (Morgan et al., 1950), omission of arginine is bound to have an adverse effect on the cells. Therefore is it possible that the balance of nutrients could be so adjusted to permit cell survival and inhibit virus outgrowth? If such a system could be devised and if, in addition, the virus could be 'recalled' at will by a further alteration in nutrient balance, this would fulfil the concept of latency as a reversible intracellular suspension of viral activity: and it would do so by a mechanism conceivable in the in vivo state.

The object of the first part of this study on latent infection of herpesvirus in tissue culture was to investigate the possibility that balance of nutrients may be one of the basic mechanisms controlling whether a virus invades a cell and continues multiplication or whether it invades a cell and initiates a latent infection.

In such an investigation it is important to consider the fate of the cellular partner of the host-virus relationship. Therefore, all findings on the effects of nutrient imbalance on virus infected cells will be considered relative to the effects on normal cells. Both will be compared with the basic work on nutritional requirements for the growth of cells in tissue culture by Eagle and his co-workers (e.g. 1955a, b,c; 1956a,b; 1961) and by Morgan et al. (1950).

Many factors have been implicated in predisposing a virus and its host to attain a state of latency. It is an attractive thought that the variety of approaches to the latent state leads ultimately to a few simple pathways and that basically only a few mechanisms operate. The aim of this work was to make a step towards finding these common pathways.

Two basic concepts, that of a reversible inactivation of virus extracellularly and that of a reversible inactivation of virus intracellularly, have been selected for study here. The experimental model is

Herpes simplex virus and rabbit kidney cells. The findings will therefore apply directly only to this system. However, it is hoped that this may serve as a framework for the understanding of latent infections of other viruses attained by other methods when these are considered from basic principles.

PART II

A Tissue Culture Model of a Latent Infection with Herpes Simplex Virus

MATERIALS AND METHODS

VIRUSES

1. Herpes simplex virus

a) Strain HFEM was obtained from Dr P. Wildy,* Institute of Virology, Glasgow, and subsequently passaged in this laboratory in HEp-2 cells to give a crude freeze-thaw lysate.

b) City Hospital strain 244 was wild strain isolated by Dr M. Moffat from a throat swab taken from a patient at the City Hospital, Edinburgh. The virus preparation used had been carried through three passages in HEp-2 cells.

c) Roizman's MP strain had been isolated and characterised by Roizman and Roane (1961, 1963). This was a cloned strain of herpesvirus which caused formation of polykaryocytes or syncytia in several types of cell culture. The virus culture used here was derived from a freeze-dried preparation supplied by Dr B. Roizman, Johns Hopkins University School of Medicine, Baltimore. This had been reconstituted in water and consequently passaged in RK_{13} cells. It will be referred to throughout this work as Herpes simplex virus (MP) or HSV (MP).

2. Vaccinia virus

The lapinised Lister strain, see Part I.

3. Coxsackie B_3

The strain used had been obtained from the Central Public Health Laboratory, Colindale, as a freeze-dried preparation and passaged twice in monkey kidney cells to give a crude freeze-

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thaw lysate.

4. Influenza PR_8

The strain used had been passaged in this laboratory for many years in the allantoic cavity of fertile eggs.

CELLS

1. RK_{13}. A continuous line of rabbit kidney epithelial cells. See Part I.

2. HeLa. A subculture of this cell line was obtained from Dr R. Postlethwaite, Department of Bacteriology, Aberdeen University. These are malignant epithelial cells of human origin.

3. HEp-2. A line of malignant epithelial cells of human origin. See Part I.

4. Chick embryo primary cultures were prepared from 10-13 day embryos.

Chick embryos were used for propagation of Herpes simplex virus and influenza virus on the chorioallantoic membrane and in the allantoic cavity respectively.

MEDIA

1. Media for Propagation of Cells

a) RK_{13} cells were propagated in Glaxo tissue culture medium '199' supplemented with 10 per cent. calf serum.

b) HeLa cells were propagated in Hanks balanced salt solution supplemented with 0.2 per cent. lactalbumin hydrolysate and 12 per cent. calf serum.

c) HEp-2 cells were propagated in Burroughs Wellcome 'Eagle's' minimum essential tissue culture medium, supplemented with 10 per cent. Difco tryptose phosphate broth, 0.05 per cent. sodium bicarbonate and 10 per cent. calf serum.

d) Medium used for chick embryo primary cultures was as for HEp-2 cells.

As in Part I serum was sterilised by seitz-filtration and inactivated by heating to 56°C for 30 minutes. All media used throughout this work contained penicillin and streptomycin to a final concentration of 100 units per ml and 100 µg per ml respectively.

2. Overlay Media for Plaque Titrations

An agar overlay was used for plaque titration of coxsackie B_3 virus. This consisted of the appropriate growth medium for the cells solidified with 1.2 per cent. Difco noble agar.

Herpesvirus and vaccinia virus were titrated using a methylcellulose (Methocel) overlay. The Methocel used was obtained from L. Light and Co., Ltd., Colnbrook, Bucks. It was incorporated into the overlay medium to a final

concentration of 0.75 per cent. Methocel overlays remain liquid throughout incubation and prevent formation of secondary plaques by localising extracellular viruses which cannot diffuse through solutions of such high viscosity. It was essential, therefore, that cultures under a Methocel overlay remained undisturbed throughout incubation.

3. Other Solutions

Other solutions were used for different purposes; for diluting biological materials, for washing cells and for other purposes. These were; Dulbecco's solution, Hank's balanced salt solution (Hanks BSS), and 1 per cent. skim milk solution. The last was used for diluting herpesvirus in routine titrations since it was found the stability of the virus was greater in this than in growth medium or a salt solution. Skim milk was prepared by reconstituting skimmed milk powder in distilled water and sterilising by tyndallisation, heating to 100°C for 30 minutes on three successive days. Skimmed milk powder was obtained from Eustace Miles Foods and Co., Ltd., Colnbrook, Bucks.

4. Experimental Media for Nutritional Studies

The Eagle's medium and variations of this used in nutritional studies were based on the formula given by Paul (1961) and was made up from the stock solutions listed below. All solutions were sterilised by filtration through Millipore filters with membranes of porosity 0.22 μ, and all stored at

-30°C. Media were made up from the stock solutions on the day of use since complete medium deteriorates on storage.

Solution 1

NaCl	8.00 gm wt	dissolved in 1 litre of distilled water
KCl	0.40 "	
$CaCl_2$	0.14 "	
$MgSO_4.7H_2O$	0.10 "	
$MgCl_2.6H_2O$	0.10 "	
$Na_2HPO_4.2H_2O$	0.06 "	
KH_2PO_4	0.06 "	
phenol red	0.02 "	

Solutions of group 2

L-arginine.HCl	0.211 gm wt	each compound dissolved in a separate 10 ml volume of solution 1
L-histidine.HCl	0.039 "	
L-isoleucine	0.262 "	
L-leucine	0.131 "	
L-lysine.HCl	0.227 "	
L-methionine	0.075 "	
L-phenylalanine	0.083 "	
L-threonine	0.119 "	
L-tryptophan	0.020 "	
L-valine	0.117 "	

Solutions of group 3

L-cystine	0.06 gm wt	each compound dissolved in separate 50 ml volumes of 0.1 N.HCl
L-tyrosine	0.18 gm wt	

Solutions of group 4

choline	0.100	gm wt	each compound dissolved in separate 100 ml volumes of solution 1
nicotinic acid	0.100	"	
pantothenic acid	0.100	"	
pyridoxal.HCl	0.122	"	
riboflavin	0.010	"	
thiamine.HCl	0.112	"	
i-inositol	0.100	"	

Solutions of group 5

biotin	0.01	gm wt	each compound dissolved in separate 50 ml volumes of solution 1 by adding 0.5 N NaOH dropwise, to adjust the pH to neutrality
folic acid	0.01	"	

Solution 6

glucose	10	gm wt	dissolved in 50 ml of solution 1

Solution 7

$NaHCO_3$	1.4	gm wt	dissolved in 100 ml of distilled water

Solution 8

Penicillin

Streptomycin

Solution 9

L-glutamine	1.46	gm wt	dissolved in 100 ml of solution 1

100 ml of complete medium was prepared by mixing the following volumes of these stock solutions.

		Total volume
group 2	0.1 ml of each	1.0 ml
group 3	0.5 ml "	1.0 ml
group 4	0.1 ml "	0.7 ml
group 5	0.5 ml "	1.0 ml
group 6	1.0 ml	1.0 ml
group 8	0.1 ml	0.1 ml
group 9	1.0 ml	1.0 ml
		5.8 ml

5.8 ml of Eagle's medium was made up to 100 ml with solution 1, and the pH adjusted to 7.2 - 7.4 with solution 7 (sodium bicarbonate). When one or more components of the medium were omitted for test purposes an equivalent volume of solution 1 was added.

The following solutions were used in some experiments on the thymine requirement of cells for the production of viruses. Stock solutions were prepared in distilled water, sterilised by filtration through Millipore filters of porosity 0.22 μ, and stored at -30°C.

Adenine sulphate	3×10^{-3} M
Thymidine	3×10^{-1} M
Glycine	3×10^{-2} M
Aminopterin	10^{-5} M

The concentrations were expressed as molarities since this made further calculations easier in these experiments.

All amino acids, vitamins, sugars, adenine and thymidine were obtained from British Drug Houses Ltd., Poole, England, with the following exceptions: leucine, riboflavin, pyridoxal and aminopterin which were obtained from L. Light and Co., Ltd., Colnbrook, Bucks.

GROWTH OF CELLS

Cells were propagated in pyrex Roux bottles fitted with silicone bungs.

Continuous cell lines were subcultured at seven-day intervals. Cell sheets were stripped from the glass using the following procedure. Nutrient medium was discarded, the cultures rinsed with Dulbecco's solution and flooded with a mixture of 1 per cent. trypsin and 0.02 per cent. versene in Dulbecco (pH 7.6) at room temperature. The trypsin-versene mixture was gently decanted after about 3 minutes and the cell sheets observed for signs of disintegration; when this was first seen 10 ml of growth medium was added to each Roux bottle and the cells dislodged by manual shaking. Clumps of cells were disaggregated by pipetting and the cells counted in a haemocytometer. The stock of cells was then used to prepare monolayers for experimental purposes and some kept as seed for further propagation.

Chick embryo primary cultures were prepared from embryos which had been eviscerated and from which head and legs had been removed. The remaining tissue from one embryo was cut up into pieces and washed twice in Hanks BSS (pH 7.2 - 7.4) at 37°C. It then was gently shaken for 20 minutes at 37°C in 0.25 per cent. trypsin and cell clumps further disintegrated by pipetting for 1-2 minutes. The larger pieces of tissue were allowed to settle out and the supernatant decanted and centrifuged to deposit the cells. The deposit was resuspended in trypsin and the whole procedure

repeated twice. Finally the deposited cells were resuspended in growth medium and counted.

Monolayers were prepared in 8 cm pyrex Carrel flasks fitted with silicone bungs, in 6 cm pyrex petri dishes, in 1.2 cm by 15 cm pyrex tissue culture tubes with white rubber bungs or on 20 mm x 5 mm cover slips contained in cotton-wool plugged Wasserman tubes. Cultures prepared were in the growth medium appropriate for the cells. Inocula to give confluent monolayers on overnight incubation were: 6×10^6 HeLa or RK_{13} cells in 5 ml medium for Carrel flasks, 3.5×10^6 HeLa cells or 3.75×10^6 RK_{13} cells in 5 ml medium for petri dishes. Tubes and Wasserman tubes were inoculated with 1 ml volumes containing 10^5 HeLa, HEp-2 or RK_{13} cells. The former cultures were incubated stationary overnight and then with rolling for a further day to produce monolayers. The latter cultures were incubated stationary until the cells had grown into colonies rather than confluent monolayers. This usually took two to three days. Chick embryo monolayers were prepared in Roux bottles by inoculating 100×10^6 cells per bottle or in petri dishes using 10×10^6 cells per culture.

Culture vessels that were not sealed, namely petri dishes and Wasserman tubes, were incubated in an atmosphere of air enriched with about 5 per cent. carbon dioxide (CO_2) to maintain a culture pH of 7.2 - 7.4. This was carried out in an incubator continually flushed with a mixture of air, from a compressed air supply, and CO_2 from a cylinder. The mixture was passed sequentially through one Dreschel bottle containing

a copper sulphate solution to reduce contamination, through another bottle containing a phenol red pH indicator and finally allowed to play on the surface of a water reservoir inside the incubator to humidify the internal atmosphere and reduce evaporation of medium from cultures.

Unless otherwise stated all incubation was at 37°C.

PRESERVATION OF CELL STOCKS

Throughout this work subcultures of each cell line were preserved deep-frozen as a reserve in case of contamination or other deterioration. The method used was that described by Hayflick and Moorhead (1961). Cells were removed from the glass using trypsin, centrifuged, the supernatant discarded and the cells resuspended in 10 per cent. glycerol, 10 per cent. calf serum medium, to contain 1.5×10^6 cells per ml. The suspension was dispensed in 3 ml volumes in 5 ml ampoules, the ampoules sealed immediately and stored at $4^{\circ}C$ overnight. The following day the ampoules were transferred to the $-65^{\circ}C$ deep-freeze and there stored till required. To replant the cells an ampoule was removed, thawed rapidly, the contents added to 10 ml warmed growth medium, and grown in a Carrel flask at $37^{\circ}C$. The medium was changed after 24 hours to remove the glycerol. Cells were subcultured, thereafter when necessary.

PREPARATION AND STORAGE OF VIRUS STOCKS

1. Herpes simplex virus

Most experiments with Herpes simplex virus were carried out using a stock of the Roizman macro-plaque forming strain (HSV(MP)) prepared in RK_{13} cells. The virus was propagated by inoculating Roux bottle cultures of cells with HSV(MP) at a multiplicity of infection of about 1 pl. f. u. per cell. The inoculum volume was 2-5 ml and adsorption was for 3 hours at 33°C: 50 ml of growth medium was then added to each culture and incubation at 33°C continued until extensive cytopathic effect had developed. This usually took 24 hours but sometimes 48 hours. If the cell sheet still adhered to the glass most of the growth medium was gently decanted and discarded. The cells were then shaken off the glass into the remaining small volume of liquid (about 10 ml). If, however, the cell sheet had completely disintegrated under virus action, the contents of the bottle were harvested as a whole. Virus was liberated by ultra-sonic disruption of cells using an M.S.E. ultra-sonic disintegrator at maximum frequency (20 Kc per sec) for 3 minutes. The apparatus used was of the type with a probe which dipped into the preparation to be treated. The infected cell suspension was contained in a thick-walled, round-bottomed tube, fitted with a rubber cap through which the probe passed. The system was thus closed and minimised the risk of production of infective aerosols. During ultra-sonic treatment the tube containing the sample was surrounded by an

ice-bath. This treatment disrupted most of the cells releasing infective virus.

The virus preparation was titrated and dispensed in exactly 0.5 ml volumes in 0.5 dram glass bottles fitted with bakelite caps. These bottles were supplied by Johnston and Jorgenson, Ltd., London. Virus was stored at -65°C and each experiment started from one bottle of stock virus.

It was found however, that even at -65°C the titre of the virus fell, and so it was necessary at intervals to discard the stock and prepare a new batch.

2. Vaccinia virus

Preparation of the stock is described in Part I. This preparation had been partially purified from cellular material by differential centrifugation in sucrose solutions.

3. Coxsackie B_3 virus

This stock was prepared by infecting HEp-2 cells in Roux bottles at unknown multiplicity, incubating at 37°C and harvesting when widespread cytopathic effect was observed. Virus was liberated from cells by three cycles of freezing and thawing and titrated by the plaque method on HeLa cells using an agar overlay. It was dispensed in 0.5 ml volumes in glass bottles as for other viruses. Stock was stored at -30°C as it was found to be stable at this temperature.

STANDARD TITRATION TECHNIQUES

1. Plaque Titration Technique

Monolayers of cells were prepared in petri dishes. The medium was removed and cultures inoculated with a 0.5 ml volume of a suitably diluted virus suspension. In routine titrations the diluting fluid was 1 per cent. skim milk. The inoculum was distributed over the cell sheet by gentle rocking and virus adsorption continued for 3 hours at 37°C. Overlay medium containing either agar or Methocel was added, 10 ml per culture, without removing residual inoculum. Incubation at 37°C was continued for a total of 48 hours in vaccinia titrations, and for 60 hours in herpesvirus and coxsackie B_3 titrations. Cell sheets were stained before plaques were counted. Methocel overlays were removed and cultures stained with a 0.1 per cent. solution of methyl violet in saline for about 3 minutes to render plaques more clearly visible. To stain cultures which had agar overlays, 3 ml of an agar medium containing neutral red at a final dilution of 1:20,000 was added at 48 hours and incubation continued for a further 12 hours to allow the stain to diffuse through to the cells.

Counts of 50-150 plaques per petri dish were considered optimal since numbers less than this had a large percentage variation and numbers much greater resulted in the plaques becoming semi-confluent and eventually confluent. Titrations were comparable only when conditions were kept constant. In particular the adsorption period had to be fixed since virus

adsorption was incomplete after 3 hours, a standard proportion of virus only being adsorbed in this time. All titrations were carried out in duplicate and results were expressed as plaque forming units per millilitre (pl. f. u. per ml).

2. Tube Titration Technique

Tube cultures of cells were prepared as described above. Growth medium was removed and cell monolayers inoculated with a 0.1 ml volume of virus diluted in 1 per cent. skim milk. On some occasions it was necessary to use another diluting fluid; this is recorded where appropriate. Dilutions were usually inoculated into five or six replicate tubes. Virus adsorption was for 3 hours at 37°C with rolling, after which 0.8 ml of growth medium was added to each culture and incubation with rolling continued. Cultures were examined microscopically for viral cytopathic effect after a stated interval, and the titre of the virus preparation calculated as a 50 per cent. tissue culture infective dose ($T.C.D._{50}$) according to the method described by Reed and Meunch (1938).

EXPERIMENTS ON THE NUTRITIONAL REQUIREMENTS OF CELLS TO SUPPORT VIRUS GROWTH

Preparation of cell monolayers has been described. Before cultures were inoculated for nutritional studies, growth medium was removed and the cell sheets washed twice with Hanks BSS (pH 7.2) to remove most of the extracellular nutrients, namely sugars, proteins, amino acids, growth factors, etc. Cultures were finally drained and inoculated with virus suitably diluted in Hanks BSS. Volumes of inocula were 0.5 ml for Carrel flasks and petri dishes and 0.2 ml for tube cultures and tubes containing cover slips. Adsorption was usually for 2 hours at 37°C with intermittent gentle rocking. Residual inoculum fluid was then drained off and the cultures washed a further twice with Hanks BSS to remove unadsorbed virus. Experimental media were added, incubation continued at 37°C and cultures withdrawn according to the requirements of the test.

Variations on this procedure will be recorded where appropriate.

Various effects of the balance of nutrients were investigated.

1. The effect of nutrition on virus yield was examined in Carrel flask, petri dish and tube cultures. In experiments in flasks and petri dishes, the medium was removed and assayed for virus yield where necessary; 3 ml of sterile distilled water was added to each infected cell sheet and cells scraped off using a silicone tipped 'policeman'. The cells were removed,

further lysed by three cycles of freezing and thawing and the virus assayed. Tube cultures were harvested complete by three cycles of freezing and thawing and then titrated.

2. The effect of nutrition on plaque formation was investigated by incorporating Methocel or agar, according to the virus, into the test medium and then incubating and staining as in a standard plaque titration. This was carried out mainly in petri dishes but sometimes in Carrel flasks.

3. Development of gross viral cytopathic effect was observed by microscopic examination of tube cultures.

4. Formation of viral inclusions under different nutritional conditions was studied by Giemsa staining of infected cover slip cultures. This was carried out at room temperature. The following was the method used.

a) Cover slip cultures were rinsed with phosphate buffered saline at pH 7.2;
b) treated with Shaudin's fixative for 30 minutes at room temperature;
c) rinsed again with the buffer;
d) stained overnight with a 1:60 dilution in buffer of Giemsa's stain;
e) rinsed in distilled water;
f) differentiated with colophonium in methanol, 2 drops in 50 ml, by immersing for 10 seconds;

g) dried at room temperature;

h) dipped in xylol, mounted in Canada balsaam and examined.

5. Production of viral deoxyribonucleic acid and changes in the cell nucleus were studied on infected cover slip cultures by staining with acridine orange. This procedure was carried out at room temperature. The following was the method used.

a) Cover slip cultures were fixed for 5 minutes using a 2:1 mixture of glacial acetic acid and absolute ethanol;

b) washed twice, over a period of 2 minutes, in a citrate-phosphate buffer at pH 3.8;

c) stained for 5 minutes with a 0.1 per cent. acridine orange solution (the stain had been freshly prepared from a 1 per cent. distilled water solution by a further 1:10 dilution in the buffer);

d) washed twice, over a period of 2 minutes, in the buffer;

e) mounted in buffer and the edges of the cover slip sealed with nail polish to prevent drying;

f) and examined under ultra-violet illumination.

Using this stain deoxyribonucleic acid fluoresces green and ribonucleic acid fluoresces red.

6. The production of virus protein was investigated by two methods. Firstly the fluorescent antibody staining technique

was used to detect viral antigen within the cell and secondly the complement fixation test was used to detect virus-induced soluble complement fixing antigen.

The fluorescent antibody staining technique was carried out on infected cover slip cultures. This procedure involved treating herpesvirus-infected RK_{13} cells with human anti-herpes serum and subsequently with fluorscein-conjugated anti-human globulin. As a precaution to reduce non-specific fluorescence the human serum was adsorbed with a powder prepared from uninfected RK_{13} cells. Uninfected cells were ground in acetone and the residue dried to produce the cell powder. To remove material from the serum which might produce non-specific staining with the infected RK_{13} preparation 100 mgm of powder was mixed with 1 ml of serum for 30 minutes at room temperature and then centrifuged out. The conjugated globulin used was Bacto FA antibody goat anti-rabbit globulin. As before, staining was at room temperature. The following was the method used.

a) Cover slip cultures were fixed by immersion in acetone for 10 minutes;

b) dried in air;

c) stained for 30 minutes with absorbed human antiserum;

d) rinsed for 10 minutes in phosphate buffered saline, at pH 7.1, with frequent agitation to remove unadsorbed antibody;

e) stained for 30 minutes with fluorescein-conjugated anti-human globulin;

f) washed in the buffer for 20 minutes with frequent agitation;

g) mounted in a 9:1 mixture of glycerol and buffer and examined under ultra-violet illumination.

With this stain there is a background of pale green uninfected cells. Infected cells in which virus protein has been synthesised fluoresce a bright apple green and this stands out from the background by virtue of the intensity of fluorescence. It is important in this method to use fluorescence-free microscope slides and immersion oil.

The complement fixation test was carried out on samples prepared by water-lysing infected cells as for assay of virus yield. Tests were carried out in WHO plates. Doubling dilutions of the antigen preparation in veronal buffered saline were made leaving a final volume of 0.1 ml per well. A complement preparation was titrated and diluted in the buffer to give 2 MHD per 0.1 ml (MHD = minimum haemolytic dose). To each well was added 0.1 ml of complement and 0.1 ml of a previously standardised human serum. The plates were held overnight at 4°C to allow fixation. The haemolytic system, sheep red cells sensitised with rabbit antiserum, was added, 0.1 ml per well. Controls were: an antibody control without antigen, an antigen control without antibody, to show that fixation was specific, and an antibody with known antigen control.

Plates were incubated at 37°C and the results read. Fixation of complement was indicated by the absence of

haemolysis of the haemolytic system.

Samples for examination by electron microscopy were prepared by infecting cells in petri dish cultures at high multiplicities. After the required interval of incubation medium was removed from the cells and discarded. Cells were washed twice with Hanks BSS, and scraped off the glass, with a silicone-tipped 'policeman', and then fixed. A double fixation method was used as this was known to give sharp resolution. Firstly cells were put into a 5 per cent. solution of gluteraldehyde in phosphate buffered saline (pH 7.2 - 7.4) and stored overnight at 4°C. The fixed cells were washed three times with a solution of 0.2 M sucrose in phosphate buffer and kept in the final sucrose solution until it was convenient to carry out the next stage, i.e. fixation in Palade's solution.

Palade's fixative was prepared from three stock solutions as below.

a) Buffered stock solution (0.28 M)
   sodium veronal 2.88 gm wt
   anhydrous sodium acetate 1.15 gm wt
   distilled water 100 ml

b) Hydrochloric acid (0.1 N)
   concentrated hydrochloric acid (36 per cent.) 8.6 ml
   distilled water 1000 ml

c) Stock osmium tetroxide (2 per cent.)
   crystalline osmium tetroxide 2 gm wt
   distilled water 100 ml

A working solution of Palade's fixative was prepared thus:

Buffered stock solution	2 volumes
0.1 N hydrochloric acid	2 volumes
Distilled water	1 volume
2 per cent. osmium tetroxide	5 volumes

The resultant solution had a pH of 6.8 - 7.6. This was stored at 4°C but discarded if it became discoloured.

The sucrose washing solution was removed from the cells and they were suspended in Palade's fixative for 20 minutes. To dehydrate, the cells were taken sequentially through 30, 50 and 70 per cent. acetone, being held for 10 minutes at 4°C in each solution; then through 95 per cent. acetone and finally through two changes of absolute acetone being held in each for 10 minutes at room temperature. The cells were embedded in an Araldite mixture which was prepared from the following materials:

Araldite epoxy resin CY212

Araldite accelerator DY064

Hardener HY964

These preparations were obtained from CIBA (A.R.L.) Ltd., Duxford, Cambridge, England.

Butyl phthalate was obtained from British Drug Houses Ltd., Poole, England.

The gelatin capsules in which the samples were embedded were size no. 00 and were obtained from Eli Lilly and Co., Indianapolis, U.S.A.

The Araldite mixture was prepared by mixing:

1 part Araldite
1 part hardener } to give a total of 19 ml

6 parts accelerator
20 parts butyl phthalate } to give 1 ml

These two preparations were mixed immediately prior to use.

The dehydrated cells were transferred to a 1:1 acetone-Araldite mixture for 30 minutes at room temperature, then to the Araldite mixture for 1 hour at 37°C and finally added to a gelatine capsule which contained a drop of Araldite. The capsule was filled with Araldite mixture and labelled. It was left at 37°C overnight, and finally polymerised to harden by incubating at 60°C for 3-4 days. The hardened blocks were trimmed and sections cut on a Porter-Blum microtome using glass knives and collected on Athene type 483 copper grids with no supporting membrane. Sections for examination were between 600 Å and 900 Å thick and were free from striations caused by faulty knives.

Sections were stained with a lead citrate-uranyl acetate stain. Lead citrate was prepared according to the method of Reynolds (1963). The uranyl acetate was a saturated solution in 70 per cent. ethanol prepared immediately before use.

The staining procedure was as follows. Grids were floated, sections down, for 2 minutes on the lead citrate solution; they were then held in 0.02 N sodium hydroxide for 20 seconds, in distilled water for 10 seconds, stained with uranyl acetate for 10 minutes and finally immersed in

50 per cent. ethanol for 20 seconds.

Sections were examined on the electron microscope, type A.E.I. E.M. 6, with an accelerating voltage of 50 kV and a resolution of 10 Å.

HEPARIN STUDIES

The heparin preparation used was Pularin, produced by Evans Medical Ltd., Speke, Liverpool. This contained 5000 I.U. per ml. Protamine sulphate (ex-herring) was obtained from L. Light and Co., Ltd., Colnbrook, Bucks. A solution containing 20,000 µg per ml was prepared in Hanks BSS (pH 7.2) and sterilised by filtration. Both solutions were stored at 4°C.

The Anti-viral Action of Heparin

Firstly the extracellular effect of heparin was investigated. These tests were carried out in cotton-wool stoppered pyrex Wasserman tubes. Heparin was diluted in Hanks BSS to give the range of dilutions to be tested and the virus suspension also diluted in Hanks BSS. In a Wasserman tube 1 ml of the heparin dilution under test and 1 ml of a standard virus dilution were mixed and held at room temperature for 15 minutes with frequent manual agitation. Residual infective virus in 0.5 ml volumes of the test mixture was then assayed by the standard plaque titration technique. In some experiments, the diluent, the duration of the experiment, or the temperature of the reaction was changed; where this was so, exact experimental conditions will be stated.

Secondly to assess the effect on later stages of the virus/host cell interaction, an appropriate concentration of heparin was incorporated in the overlay medium and added to infected petri dish cultures at the end of the normal 3 hour adsorption

period. In these cases the overlay was as normal for the cells in all respects except that heparin was an additional component. Since plaque formation of vaccinia virus and herpesvirus, HSV(MP), occur mainly by intracellular spread of virus, suppression of plaques in this experiment would indicate an intracellular anti-viral action of heparin.

INTERFERENCE EXPERIMENTS

Interference experiments between herpesvirus HSV(MP) and vaccinia virus were carried out in petri dish or tube cultures of RK_{13} cells. The ability of HSV(MP) to interfere with vaccinia was investigated, and also the ability of live, ultra-violet inactivated, and heat inactivated vaccinia virus to interfere with HSV(MP). Ultra-violet inactivated vaccinia virus was prepared by diluting stock virus in skim milk to contain 10^7 pl. f. u. per ml, and exposing 3 ml of this in a 6 cm glass petri dish for 30 seconds, 12 cm below an ultra-violet source. Vaccinia was heat inactivated by holding for 45 minutes at 56°C. Galasso and Sharp (1964) showed that such heat inactivated vaccinia could cause homologous interference. The interfering virus was added to the culture, and incubated for 2 hours at 37°C. The residual inoculum fluid was removed, the challenge virus added and incubation continued for a further 3 hours at 37°C. Growth medium was added to the cultures without removal of the second inoculum, and incubation at 37°C continued for the appropriate period. Inocula for petri dish cultures were 0.5 ml, and adsorption was with intermittent rocking. Final incubation was with a 10 ml overlay of growth medium if virus yield was being investigated or with 10 ml of a Methocel overlay for plaque formation studies. For tube cultures inocula were 0.2 ml volumes, adsorption was at 37°C with rolling and final incubation was with 0.8 ml of growth medium. At the times stated in results, cultures were

examined for viral cytopathic effect or titrated for virus yield. All subsequent titrations were by the plaque technique.

CALF SERUM ANALYSIS

Two-dimensional paper chromatography was used for the identification and approximate estimation of free arginine in calf serum samples. Whatman No. 1 paper sheets were run for 18 hours with 80 per cent. (w/v) phenol containing 0.3 per cent. (v/v) ammonia. They were then dried, rotated through 90° and run for 24 hours with butanol + acetic acid + water (4 + 1 + 5 by volume). All chromatograms were run at 25°C. Amino acid spots were detected by spraying with 1-2 per cent. ninhydrin in butanol saturated with water.

The position of arginine on the test chromatogram was located by comparison with a control chromatogram of a known mixture of amino acids. An approximate quantitative estimation was made by comparing the intensity of colouration with ninhydrin of the arginine spot on the test chromatogram with that of a range of spots of known arginine concentration on another chromatogram.

Analyses of samples of different calf serum batches gave an average result of 1.57 mg per ml calf serum. In an Eagle's solution there was 0.0174 mg per ml arginine. From the above results, in a 1 per cent. calf serum arginine-free medium there was 0.0157 mg per ml arginine or arginine-like material.

PART II

A Tissue Culture Model of a Latent Infection with Herpes Simplex Virus

RESULTS

1. SELECTION OF TEST SYSTEM

Unless specifically stated otherwise, all work was carried out using the RK_{13} cell line.

A. Herpes simplex virus

The HFEM strain of Herpes simplex virus was passaged in this laboratory in RK_{13} cells and the starting material for this work was a crude freeze-thaw lysate of infected cells. This preparation caused two types of cytopathic effect on RK_{13} cell monolayers which could be differentiated easily after 4 days of incubation. The first was a rounding of infected cells to give eventually a small heap which could be distinguished from a flat monolayer of uninfected cells. The second was the formation of polykaryocytes or syncytia which were huge multinucleate masses, containing up to several hundred nuclei. Roizman and Roane (1963) described the former type of lesions as micro-plaques (mP) and the latter as macro-plaques (MP). It was found in plaque titrations with this strain that it was difficult to identify micro-plaques with confidence from the irregularities that normally occur on a cell sheet. It was much easier and more accurate to titrate a macro-plaque forming virus and so the attempt was made to prepare and purify a stock of MP virus from this strain.

When the HFEM strain was grown on the chorioallantoic membranes of fertile eggs for 3 days and harvested by grinding the membranes, the viral progeny resulting were all of the MP type. Infected RK_{13} cells produced MP progeny for the first two passages, but after this mP variants began to appear. These results are shown in Table 1.

Table 1. Variation in plaque type of HFEM strain of herpesvirus selected by propagation in eggs or in RK_{13} cells.

Serial passages	Percentage of each plaque type MP	mP
Pass 2 in RK_{13}	80	20
" 1 " eggs	100	0
" 2 " eggs	100	0
" 1 " RK_{13}	100	0
" 2 " RK_{13}	100	0
" 3 " RK_{13}	50	50

From a comparison with the findings of Roizman and Roane (1963) it seemed likely that this virus preparation contained a mixture of virus types and that the type predominating in a population depended on the kind of host cell in which the virus was propagated.

A large batch of MP HFEM virus was prepared by propagation on the chorioallantoic membranes of fertile

eggs. Membranes were harvested after 3 days incubation and ground in a 50:50 mixture of nutrient broth and glycerol to free the virus. An attempt was made to concentrate and purify this virus preparation.

To concentrate, the virus suspension was centrifuged at 57,660 g for 45 minutes to deposit it on a cushion of a 40 per cent. solution of cesium chloride in tris (hydroxymethyl) aminomethane buffer at pH 7.8. The virus containing layer was separated and purified by chromatography on a brushite ($CaPO_4.2H_2O$) column using 0.2 M phosphate buffer as eluant (Taverne et al., 1958). Although this method gave satisfactory results, in that a purified preparation of MP virus was produced, the virus recovery rate was only 10 per cent. This and the necessity to propagate the virus on eggs, made the method unsuitable for a large scale investigation.

A wild strain of herpesvirus, designated strain 244, had been passed three times in HEp-2 cells since isolation. When titrated on RK_{13} monolayers this strain produced only micro-plaques. Strain 244 was then passed in RK_{13} cells and in eggs and subsequent preparations titrated on RK_{13} monolayers. No MP variants emerged.

A subculture of the cloned MP variant of Herpes

simplex virus was obtained from Dr B. Roizman. This was passaged and titrated in RK_{13} cells and found to form only macro-plaques. On close examination some differences in plaque type could sometimes be observed but plaques were always of the MP type, and developed to about 1 mm in diameter within 60 hours. The system of HSV(MP) in RK_{13} cells proved to be easily handled and to give reproducible results. It was therefore chosen as the experimental model.

B. Vaccinia virus

The Lister strain had been used in earlier work, see Part I. It grew well in RK_{13} cells and was easy to titrate by the plaque method.

C. Coxsackie B_3 virus

The coxsackie B_3 virus preparation had been passaged twice in monkey kidney cells in this laboratory, and the starting material for this work was a crude freeze-thaw lysate. This was passed twice in HEp-2 cells and typical viral cytopathic effect, namely rounding of cells, developed. Virus was liberated from the cells by three cycles of freezing and thawing and titrated on HEp-2, HeLa, and RK_{13} monolayers under a 1.2 per cent. agar overlay. Plaques developed on HEp-2 and HeLa cultures but there was no cytopathic effect on RK_{13} cells, even at high virus concentrations.

On the two susceptible cell lines there was a big variation in plaque size in individual cultures and although the two lines were equally sensitive in terms of numbers of infective foci, plaques generally were bigger on HeLa cells than on HEp-2 cells. It was found that an agar overlay was necessary for plaque development. Under a Methocel overlay generalised cytopathic effect occurred without formation of discrete plaques. This may be a reflection on the small size of this virus as compared with herpes or vaccinia viruses or it may reflect the explosive type of virus release as opposed to slow leakage of virus from cells.

From these experiments the test system selected was coxsackie B_3 virus growing in HeLa cells, and with an agar overlay in plaque titrations.

2. NUTRITIONAL STUDIES ON VIRUS INFECTED TISSUE CULTURE CELLS

The object of the experiments to be described was to evolve a tissue culture model of a latent herpetic infection by means of removal and subsequent later replacement of single nutrients essential for the reproduction of the virus. The specificity of requirement for a nutrient was assessed by growing infected cells in a medium deficient in the nutrient under investigation and measuring the degree of suppression of virus growth under these conditions. Reversibility of suppression was indicated by a resumption of viral reproduction upon later replacement of the missing nutrient.

A. The Effect of a Range of Nutrient Omissions on the Growth of Herpes simplex virus in RK_{13} Cells

Tankersley (1964) had investigated the effect of the systematic omission of each of the amino acid components of Eagle's medium on the growth of a strain of herpesvirus in a line of human cells. He found that the greatest specific requirements were for arginine and histidine and that the least requirement was for lysine. These three basic amino acids, covering the extremes of requirement, were selected here for initial studies. Monolayers of RK_{13} cells were prepared in

tubes, drained and inoculated with HSV(MP) using a calculated inoculum of 10, 5 or 1 pl. f. u. per tube, and 0.8 ml of 5 per cent. calf serum 199 medium added to each culture. Incubation at 37°C was continued for 24 hours and then cultures were examined for viral cytopathic effect (CPE), drained, washed twice with Hanks BSS and inoculated with 1 ml of appropriate experimental medium. After further 24 hours and 48 hours tubes were re-examined for CPE and finally cultures were harvested by three freeze-thaw cycles and the virus yield assayed by the plaque technique. The results of two such experiments are recorded in Table 2.

These results confirmed Tankersley's findings that omission of either arginine or histidine from an Eagle's medium caused a marked reduction in virus yield and that there was no such reduction upon the omission of lysine. Indeed it did appear from Table 2 that the omission of lysine resulted in a system of increased sensitivity to trace infection. Further, it was observed that viral CPE ceased to develop when arginine was removed from the medium but continued to develop when histidine was omitted.

Using a similar procedure to that of Tankersley the effect of a range of omissions on the growth of HSV(MP) in RK_{13} cells was investigated. The effect of omitting from Eagle's medium the eight water soluble

Table 2. The effect of three amino acid deficiencies on the growth of HSV(MP) in RK_{13} cells. 1. and 2. are repeat experiments using three different inocula, (a), (b) and (c).

Experimental medium	Formation of viral CPE after: 24 hr in 5 per cent. calf serum 199		a further 48 hr in exptl. medium		Final virus yield in pl.f.u. per tube	
	1.	2.	1.	2.	1.	2.
(a) Calculated inoculum: 10 pl.f.u. per tube						
Eag	5/5	Trace syncytia	5/5	6/6	75,000	10,500
Lys$^-$	6/6		6/6	6/6	60,000	23,500
Arg$^-$	5/6		6/6 *	6/6 *	100	505
Hist$^-$	6/6		5/5	6/6	600	115
(b) Calculated inoculum: 5 pl.f.u. per tube						
Eag	4/6	Trace syncytia	6/6	5/6	55,000	3,400
Lys$^-$	5/6		6/6	5/6	50,000	23,500
Arg$^-$	4/6		4/6 *	3/6 *	200	665
Hist$^-$	4/6		1/2	3/6	3,000	15
(c) Calculated inoculum: 1 pl.f.u. per tube						
Eag	0/5	Trace syncytia	1/5	2/6	< 50	160
Lys$^-$	0/4		3/4	4/6	115,000	16,000
Arg$^-$	0/6		0/5	0/5	< 50	0
Hist$^-$	1/5		4/5	0/5	1,000	0

* Syncytia remained small and localised

vitamins and i-inositol was studied. The omission of glutamine was studied since Lewis and Scott (1962) and Tankersley (1964) had reported a high specificity for this nutrient in herpesvirus reproduction. Cyst(e)ine has been found by several workers (e.g. Tyndall and Ludwig, 1960), to be the most critical amino acid in the reproduction of some coxsackie and poliovirus strains. The effect of this omission on herpesvirus was examined for comparison. The final solution tested was prepared from experimental solution 1 which is a modified Hanks BSS, supplemented with glucose as a source of carbon and energy and with arginine and histidine since these have been found to be critical for the production of HSV(MP).

The calculated input inocula and virus yield in each experimentalmedium is shown in Table 3.

The results shown in Table 3 indicate that virus reproduction continued, although to a reduced extent, in the absence of the water soluble vitamins and i-inositol. It appears that there was some limited virus growth in the absence of glutamine but this test would have to be repeated several times to get a clearer result. In the remaining two media, i.e. $Cyst^-$, and Hanks + additives, there was generalised non-specific cellular deterioration so that little confidence can be placed in the final virus yields. However when these results are compared with those

Table 3. The effect of some nutrient deficiencies on the growth of HSV(MP in RK_{13} cells.

Experimental medium	Formation of viral CPE after 48 hr in exptl. medium	Final virus yield in pl.f.u. per tube
(a) Calculated inoculum: 1000 pl.f.u. per tube		
Eag	$\frac{6}{6}$	17,000
$Vits^-$	$\frac{5}{5}$	13,800
$Glut^-$	$\frac{6}{6}$	10,400
* Cys^-	$\frac{6}{6}$	3,680
* Hanks + additives	$\frac{5}{5}$	1,980
(b) Calculated inoculum: 100 pl.f.u. per tube		
Eag	$\frac{6}{6}$	8,800
$Vits^-$	$\frac{5}{6}$	2,920
$Glut^-$	$\frac{1}{6}$	600
* Cys^-	$\frac{5}{6}$	60
* Hanks + additives	$\frac{5}{5}$	200
(c) Calculated inoculum: 10 pl.f.u. per tube		
Eag	$\frac{2}{6}$	324
$Vits^-$	$\frac{1}{6}$	46
$Glut^-$	$\frac{0}{6}$	62
* Cys^-	$\frac{0}{6}$	8
* Hanks + additives	$\frac{0}{6}$	2

* Cell visibly deteriorated in these media, a large proportion of the population became granular.

shown in Table 2, it appears virus growth occurred to a greater or lesser extent in all media tested except Arg^- and $Hist^-$.

The suggestion, recorded in Table 2, that a lysine deficient medium was more sensitive than a complete medium to trace infection of RK_{13} cells with HSV(MP) was investigated further since this might be of value in virus isolation. According to the method described for the initial experiments, infections were established in tube cultures. Tubes were drained and washed after 24 hours infection and reincubated with either Eag or Lys^- medium.

The results of a series of tests including those abstracted from Table 2 are shown in Table 4.

The results in Table 4 show that, in 9 tests out of 12, HSV(MP) grows as well or slightly better in a lysine deficient medium than in complete medium. In only three tests was the increased yield in deficient medium really significant. These results suggested that the 100-fold or greater increase in virus yields was not a reproducible occurrence and that when considered against the adverse effect on cells it was not advantageous to use a lysine deficient medium in routine isolation of herpesvirus.

Following the work of Pollard and Sharon (1963) on

Table 4. Comparative yields of HSV(MP) from RK_{13} cells in Eagles and in lysine deficient media.

Virus yield in pl.f.u. per tube culture of cells in:	
Eag	Lys^-
75,000	60,000
10,500	23,500
55,000	50,000
3,400	23,500
< 50	115,000
160	16,000
220	920
154	200
76	20
920	1,540
290	310
78	132

prolonged latency induced in psittacosis-infected cells by aminopterin, the effect of restriction of thymine metabolism on herpesvirus growth was investigated. The action of aminopterin can be limited to the thymine pathway if it is used in conjunction with adenine and glycine (Loh, 1960). The effect of such a system on the plaque formation of HSV(MP) and vaccinia viruses in RK_{13} cells was investigated.

Monolayers of cells were prepared, drained, washed with Hanks BSS, and inoculated with virus. After a 3-hour adsorption period, residual inoculum was removed, the cultures washed again and overlaid with experimental media. All media were based on Eagle's solution and contained Methocel to a final concentration of 0.75 per cent. Adenine sulphate was used at a final concentration of 3×10^{-5} M, glycine at 3×10^{-4} M, thymidine at 3×10^{-5} M and aminopterin at 5×10^{-7} M. Vaccinia cultures were incubated for 48 hours and herpesvirus cultures for 60 hours, both at 37°C, and subsequently examined for plaque formation.

Results obtained are shown in Table 5.

The results shown in Table 5 indicate that aminopterin at a concentration of 5×10^{-7} M greatly reduced plaque formation by vaccinia and herpesvirus both in plaque size and number, and that this reduction was specifically reversed by the addition of thymidine. It was also observed that plaque size of HSV(MP) was

Table 5. The effect of aminopterin on plaque formation by HSV(MP) and vaccinia viruses on RK_{13} monolayers.

Overlay medium	Number of plaques per petri dish: HSV(MP) size	Vaccinia
Eag	183 normal (N)	270
Eag + Ad + Gly + Thy	234 > N	250
Eag + Ad + Gly + Thy + AP	192 > N	210
Eag + Ad + Gly	201 > N	230
Eag + Ad + Gly + AP	72 *	60 †

* appeared only as slight irregularities on the cell sheet

† very much smaller than normal

Ad = adenine sulphate, Gly = glycine, Thy = thymidine, AP = aminopterin

considerably increased under overlays containing adenine, glycine and thymidine or containing only adenine and glycine.

From this survey it appeared that, of the media tested, that deficient in arginine supported the least virus reproduction, the least viral cytopathic effect, and did not cause non-specific cellular damage over the short period of test. The effect of the presence or absence of arginine in a nutrient medium on uninfected and HSV(MP)-infected RK_{13} cells was investigated further.

B. The Effect of an Arginine Deficiency on Uninfected and Virus Infected RK_{13} Cells

a) The effect of an arginine deficiency on RK_{13} cells

Experiments were carried out on cells growing in Carrel flasks. Cells were normally grown in 10 per cent. calf serum 199 and the calf serum used was undialysed. Comparisons were made between cells growing in 199, in Eag and in Arg^- so that the effect of removing the cells from their normal growth medium as well as the specific effect of arginine omission could be assessed.

i. Gross Morphology.

Cells grown in Arg^- medium remained morphologically normal for 3 days. After this they became thinner and more spindle shaped and eventually fell off the glass. In other experiments, when Arg^- medium was supplemented with calf serum, cells remained morphologically normal for longer periods, even up to 16 days with 10 per cent. calf serum. With regard to morphology, some other points emerged. Cells growing in deficient media were not easily subcultured. After the action of a trypsin-versene mixture a large proportion of the cells became vacuolated or granular; sometimes they continued to grow but the resultant population contained very many abnormal and swollen

cells. In cultures which were maintained in changes of deficient medium but were not subcultured, the cells looked normal until 1-3 days before death. About this time they became thin and spindle shaped. A considerable proportion of these cells could still recover at this stage if deficient medium was removed and the optimal calf serum 199 medium replaced. The vacuolated populations resulting from the trypsin treatment, however, did not recover when optimal medium was replaced.

ii. Viability of Cells.

Viability of cells was judged by their resistance to staining with trypan blue and by their ability to multiply. Cultures were prepared in Carrel flasks, drained, washed and reincubated in experimental media. Cultures were withdrawn in pairs at intervals. Cell counts were made for each culture and the average taken of each like pair.

Viability of cells in Arg^- medium is recorded in Table 6.

The results in Table 6 show that cells in Arg^- medium can multiply to a very limited extent, this may be due to a residual intracellular pool of arginine, and that they remain viable for 3 days but die rapidly between 3 and 4 days. These results suggest that the cells may have divided between 3 and 4 days and died

Table 6. Growth of RK_{13} cells in complete and arginine-deficient media.

Incubation period in days	Number of cells per culture when grown in:					
	3 per cent. calf serum 199		199		Arg^-	
	Viable	Dead	Viable	Dead	Viable	Dead
0	1.0×10^6		1.0×10^6		1.0×10^6	
1	1.6×10^6	$.18 \times 10^4$	1.2×10^6	9×10^4	1.7×10^6	5×10^4
2	4.8×10^6	4×10^4	3.6×10^6	3×10^4	2.2×10^6	11×10^4
3	3.9×10^6	$.40 \times 10^4$	3.9×10^6	$.35 \times 10^4$	1.3×10^6	10×10^4
4	4.7×10^6	$.14 \times 10^4$	2.8×10^6	3×10^6	0.3×10^6	4.5×10^6

during this or soon after. These results also indicate that the cells do not grow well even in complete medium in the absence of calf serum. It was clear from this that experiments on HSV(MP) in RK_{13} cells in Arg^- medium would have to be of 72 hours maximum duration unless conditions could be adjusted to improve cell survival.

The survival of cells in Arg^- medium supplemented with calf serum was investigated. A comparison of the growth of cells in 199, Eag and Arg^-, each supplemented with 5 per cent. calf serum, is shown in Table 7.

It can be seen in Table 7 that cells grew almost as well in 5 per cent. calf serum Arg^- as in 5 per cent. calf serum Eag and only slightly more slowly than in 5 per cent. calf serum 199. The proportion of cells dying was small and approximately equal in the three media. After 3 days samples from the Eag and the Arg^- sets were subcultured and grown for a further 5 days. Cells in 5 per cent. calf serum Arg^- produced a yield only one-third as great as that from 5 per cent. calf serum Eag. Cells were morphologically normal. On a second subculture cells in the Eagle's medium continued to grow slowly but those in the deficient medium did not thrive. Only a small proportion of the latter adhered to the glass; they became vacuolated and granular and had to be discarded after a few days. In this experiment cells had been grown in 5 per cent. calf serum Arg^- for 9 days including one subculture and had

Table 7. Growth of RK_{13} cells in media containing normal or restricted amounts of arginine.

All media were supplemented with 5 per cent. calf serum.

Incubation period in days	Number of cells per culture when grown in:		
	199	Eag	Arg^-
0	1.5×10^6	1.5×10^6	1.5×10^6
1	3.4×10^6	2.5×10^6	2.1×10^6
2	5.6×10^6	4.6×10^6	2.8×10^6
3	7.4×10^6	5.1×10^6	6.2×10^6
	1st subculture, and inocula were:		
3		2.5×10^6 †	3.1×10^6 †
⋮		⋮	⋮
9		3.3×10^6	1.4×10^6
	2nd subculture, and inocula were:		
		* 0.7×10^6	* 0.7×10^6

† Cells appeared healthy and gradually multiplied in these cultures, although multiplication was much slower in the arginine-deficient medium.

* Cells did not thrive on the second subculture, they became vacuolated and granular and were discarded after 8 days incubation.

remained morphologically normal throughout.

In another experiment using 5 per cent. calf serum Arg^- these findings were confirmed, but in this case the cells were only subcultured once. They were subcultured after 4 days and incubated for a total of 19 days in deficient medium. At the end of this period, cell morphology was abnormal, the cells being very thin and reduced in size. When 10 per cent. calf serum 199 was added to these cultures and incubation continued for a further 7 days confluent monolayers resulted.

The effect of a 10 per cent. supplement of calf serum was investigated and the results obtained are shown in Table 8.

Table 8 shows the comparative growth of cells in 199, Eag and Arg^- media supplemented with 10 per cent. calf serum. The counts obtained after 1 day suggest that the cultures had almost completed one division cycle when the experimental media were added. Cells multiplied no further in the arginine deficient medium. After 4 days samples from each set were subcultured and flasks inoculated with 1.5×10^6 cells per culture. The 199 cultures grew well, the Eag cultures grew slowly, but the Arg^- cultures became vacuolated and by day 9 these cultures were dead.

In a further experiment using 10 per cent. calf serum Arg^- medium, in which cultures were examined for morphological changes but were not counted, cells

Table 8. Growth of RK_{13} cells in media containing normal or restricted amounts of arginine.

All media were supplemented with 10 per cent. calf serum.

Incubation period in days	Number of cells per culture when grown in:		
	199	Eag	Arg^-
0	1.3×10^6	1.3×10^6	1.3×10^6
1	4.9×10^6	5.0×10^6	4.1×10^6
2	4.1×10^6	4.0×10^6	1.8×10^6
3	6.0×10^6	3.0×10^6	1.2×10^6
4	6.8×10^6	5.3×10^6	1.9×10^6
	1st subculture, and inocula were:		
4 ↓ 7	1.5×10^6 grew to form mono-layer	1.5×10^6 became granular	1.5×10^6 deteriorated and culture died

appeared normal for at least 16 days. However it was clear from the density of the population that cells were dying during the experiment and it was probable that dead cells liberated arginine and other nutrients and so influenced the growth of other cells analogous to the action of cell feeder layers (Stoker and Sussman, 1965).

It appears, therefore, to be difficult to control nutrient balance in these experiments for longer than about 4 days although cells can metabolise and retain their viability for periods much longer than this.

b) The effect of an arginine deficiency on Herpes simplex virus (MP) growing in RK_{13} cells

i. The Requirement for Arginine for the Replication of HSV(MP) in RK_{13} cells.

Preliminary experiments showed that cultures deficient in arginine produced very low virus yields. These experiments were carried out in cultures where the multiplicity of infection at time of onset of deficient conditions was unknown and with the time of virus assay arbitrarily chosen. The question remains as to whether the virus continues to grow at a reduced rate or in a reduced number of cells, or whether the virus inoculum fails to complete even one replication cycle in arginine-free medium. To investigate these possibilities growth curves of HSV(MP) in RK_{13} cells infected at low and at high multiplicities were carried out. Calf serum supplemented media were used in many of these experiments to improve conditions for the cells. The effect of calf serum in deficient media on the growth of herpesvirus will be elaborated later.

A growth curve of virus in cells infected at a low multiplicity was carried out by preparing, washing and inoculating petri dish monolayers in the usual way. The inoculum was 37 plaque forming units per culture. After the 3 hours adsorption period at 37°C, cultures were drained, washed twice and then reincubated at 37°C

in experimental media. The media used were Eag and Arg^- both supplemented with 10 per cent. calf serum. Samples were withdrawn for virus assay at 3 hourly intervals for 30 hours and a final sample taken at 72 hours. Supernates were removed and assayed for virus separately. Infected cell sheets were lysed, each with 5 ml distilled water followed by three freeze-thaw cycles, and then assayed for infective virus. One set of cultures received overlays containing Methocel and were incubated for 60 hours for plaque formation.

The results obtained are shown in Figure i.

It was found that at low multiplicities of infection the virus entered eclipse by 3 hours post-infection in both Eag and Arg^- cultures. Throughout the experiment no infective virus was detected in either the supernates or cell lysates of cells growing in arginine deficient medium. In the cultures in Eagle's medium, new infective virus was first detected in the cell lysates at 15 hours post-infection and in the culture supernates 30 hours post-infection. The results of this multiple growth curve suggest that at low multiplicities of infection, herpesvirus in cells maintained in arginine deficient medium enters eclipse but proceeds no further and does not even complete one replication cycle.

A one step growth curve was carried out on infected cells growing in Eag or Arg^- medium with no

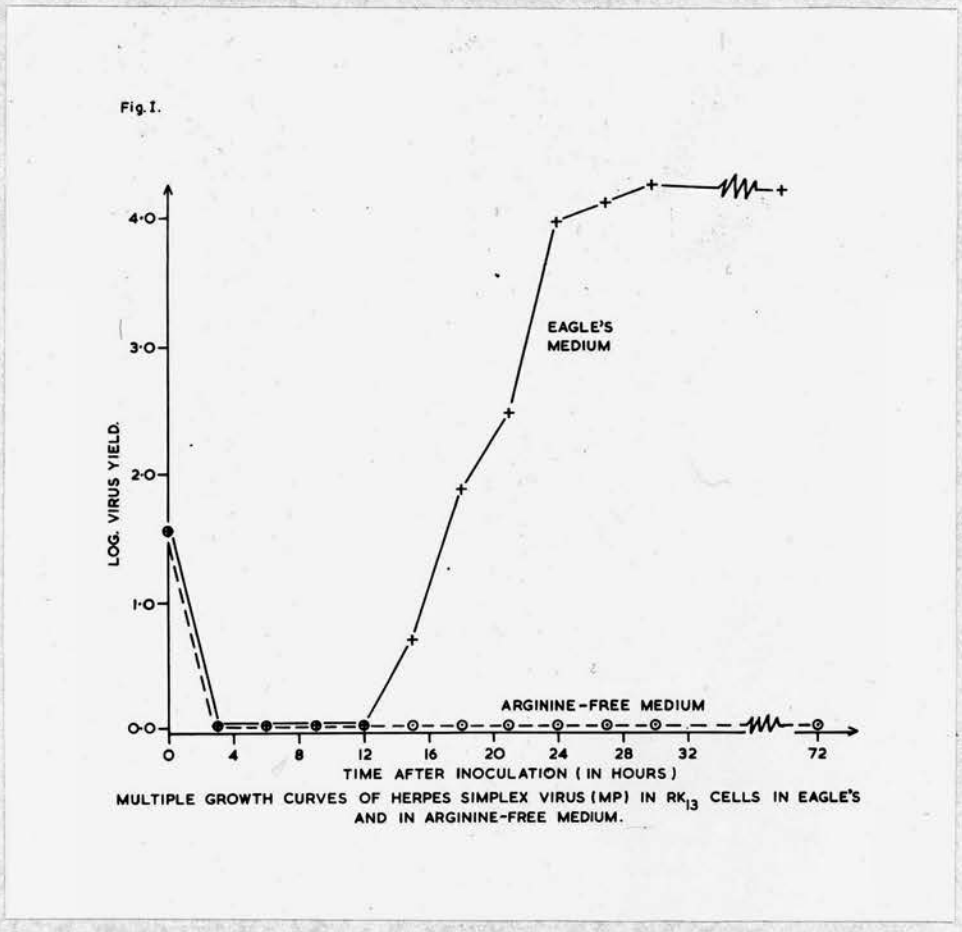


Figure i. Multiple growth curves in complete and deficient media to show the requirement for arginine for the growth of Herpes simplex virus (MP).

calf serum. Cultures were prepared, drained and washed. They were then cooled to 4°C and inoculated with virus at this temperature. Adsorption was for one hour at 4°C. Farnham and Newton (1959) reported that herpesvirus can attach to cells at this temperature but is unable to penetrate and so by this means some degree of synchrony is achieved. After adsorption cultures were drained and washed twice with Hanks BSS. Experimental media, at 37°C, were added and the cultures immediately incubated. Samples were withdrawn over 23 hours at the intervals indicated on Figure ii. Supernates were discarded since results as in Figure i suggested that little virus would be liberated from the cells within a 24 hour period. Infected cells were then harvested for virus assay, for assay of complement fixing antigen and for electron microscopic studies. For assay of infective virus and complement fixing antigen, infected cell sheets were lysed using 3 ml distilled water and three freeze-thaw cycles. For electron microscopic studies cell sheets were washed twice and scraped into a gluteraldehyde fixative, and treated as described on page 115. These samples and those for assay of complement fixing antigen will be considered later. The multiplicity of infection was of the order of 1 pl. f. u. per cell but it has become evident during these studies that recovery of infective virus from cells is always less than anticipated. The

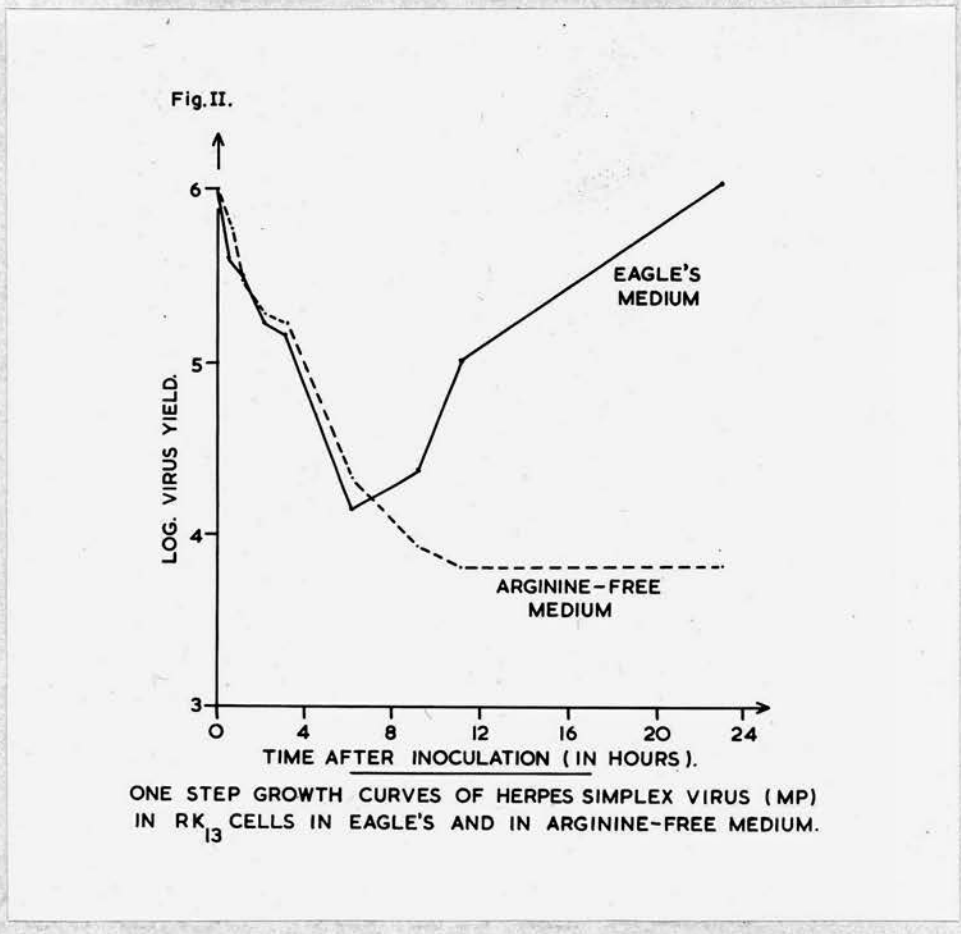


Figure ii. One-step growth curves in complete and deficient media to show the requirement for arginine for the growth of Herpes simplex virus (MP).

multiplicity may therefore have been greater than one. The number of physical particles attached to each cell was certainly much greater than one. Infective virus recovered from the cells was titrated by the plaque technique and the results obtained are shown in Figure ii.

Results shown in Figure ii suggest that attached virus penetrates the cells and that viral infectivity is eclipsed at equal rates in a complete medium and in an arginine-free medium. In the deficient medium the virus does not complete one growth cycle.

ii. Adsorption of Virus in Arginine-free Medium.

Virus adsorption to cells from an inoculum in a balanced salt solution was observed in preceding experiments but it was of interest to know whether the rate of virus attachment to cells was the same in the deficient as in the complete medium.

Adsorption of virus from 199 and from Arg^- medium as diluting fluids was compared. Monolayers of cells were prepared, drained and washed with Hanks BSS. Virus inoculum in 3 ml of either 199 or Arg^- medium was added. At the intervals shown in Table 9 cultures were drained and a 10 per cent. calf serum 199 overlay containing Methocel added. Cultures were always treated in pairs. After 3 hours adsorption two additional cultures from the Arg^- set were overlaid with 10 per cent. calf serum Arg^- medium containing Methocel.

Cultures were incubated for 60 hours at 37°C and then examined for plaque formation.

Results obtained are shown in Table 9.

Table 9. Adsorption of HSV(MP) to RK_{13} cells in arginine-free medium.

Adsorption period in hours	Number of plaques formed per culture when inoculum was diluted in: 199	Arg^-
1	52	41
2	44	56
3	46	59
3	Adsorption in Arg^- and incubation in Arg^- resulted in no plaques being formed	

Results shown in Table 9 indicate that virus can be adsorbed on to cells in an arginine-free medium and that the rate of attachment does not depend on the presence or absence of arginine in an Eagle's medium. There was no plaque formation when adsorption and incubation was in an arginine deficient medium. In subsequent nutritional experiments adsorption was always from Hanks BSS.

iii. Reversibility of an Arginine Deficiency-induced Virus Suppression.

Since the object of these experiments was to produce a model of a latent infection in tissue culture,

the next question to be considered was whether the cessation of virus growth was irreversible or whether replacement of arginine in a deficient system initiated a resumption of viral replication.

This was investigated initially in the following experiment. Petri dish cultures were prepared and inoculated with virus after appropriate washing. These cultures were then maintained in 10 per cent. calf serum Arg^- medium. After increasing intervals of time the deficient medium was withdrawn and replaced with 10 per cent. calf serum Eag medium and incubation continued for a further 48 hours. Cultures were finally examined for plaque formation. Results obtained are shown in Table 10.

Table 10. Survival of 'latent' HSV(MP) in RK_{13} cells as potentially infective foci.

Incubation conditions	Number of plaques per petri dish
*Eag for 72 hours	approx. 500
Arg^- for 72 hours	0
Arg^- for 24 hours then Eag for 48 hours	approx. 400
Arg^- for 48 hours then Eag for 48 hours	293
Arg^- for 72 hours then Eag for 48 hours	143

* All media contained 10 per cent. calf serum and 0.75 per cent. Methocel.

From these results it was seen that virus resumed growth after a period of suppression caused by an arginine deficiency. Furthermore plaque size attained after 48 hours in complete medium was constant irrespective of the duration of the preceding suppression period, within the limits of this experiment. There was, however, a decrease in the number of plaques recovered and the decrease continued with time. That the 'recalled' plaques arose from the original foci of infection and not from a slow secondary spread was further investigated later.

iv. The Growth of HSV(MP) in RK_{13} Cells in Media Containing Restricted Concentrations of Arginine.

Studies on the survival of cells in total absence of arginine have shown that cells die off rapidly after 3 days. An attempt was made, therefore, to adjust conditions so that the survival time of cells was extended but so that they were still unable to support the replication of herpesvirus. It was observed that RK_{13} cells did not grow well in the absence of calf serum even in their normal growth medium. Therefore it was decided to investigate the effect of addition of calf serum to an arginine-free medium on HSV(MP)-infected RK_{13} cells. The calf serum used was undialysed so it was hoped by this means to introduce trace amounts of

arginine to the system and concomitantly to avoid the other undetermined adverse effects on cells caused by calf serum omission.

The effect of a range of calf serum concentrations on plaque formation and on virus yield of HSV(MP) in RK_{13} cells was investigated. Petri dish cultures were prepared, washed and inoculated in the usual way. Duplicate cultures then received 10 ml of test overlay containing Methocel and were incubated for plaque formation. Other cultures, for assay of virus yield, received parallel overlays but without Methocel. Cultures were harvested after 60 hours incubation at 37°C. The range of calf serum concentrations tested was from 20 per cent. to none.

Results obtained are shown in Table 11. Figure iii (a-j) is included to show the variation in plaque size which occurred with different media.

The results in Table 11 show that, at low multiplicities of infection, an $^{A}rg^{-}$ medium supplemented with up to 20 per cent. calf serum was unable to support replication of herpesvirus. As far as it was possible to see by microscopic screening of cultures there was no viral CPE in any of the Arg^{-} cultures.

A similar type of experiment was carried out using tube cultures and a high multiplicity of infection. Tube cultures were prepared, drained, washed and inoculated by the standard method. Samples were withdrawn

Table 11. Growth of HSV(MP) in RK_{13} cells in arginine deficient media supplemented with various concentrations of calf serum.

Concentration of calf serum per cent.	Number of plaques per petri dish in: Eag	Arg⁻	Number of pl.f.u. per petri dish in: Eag	Arg⁻
20	153	0	22,200	0
10	129	0	16,600	0
5	123	0	10,800	0
1	77	0	2,700	0
0	28	0	110	0

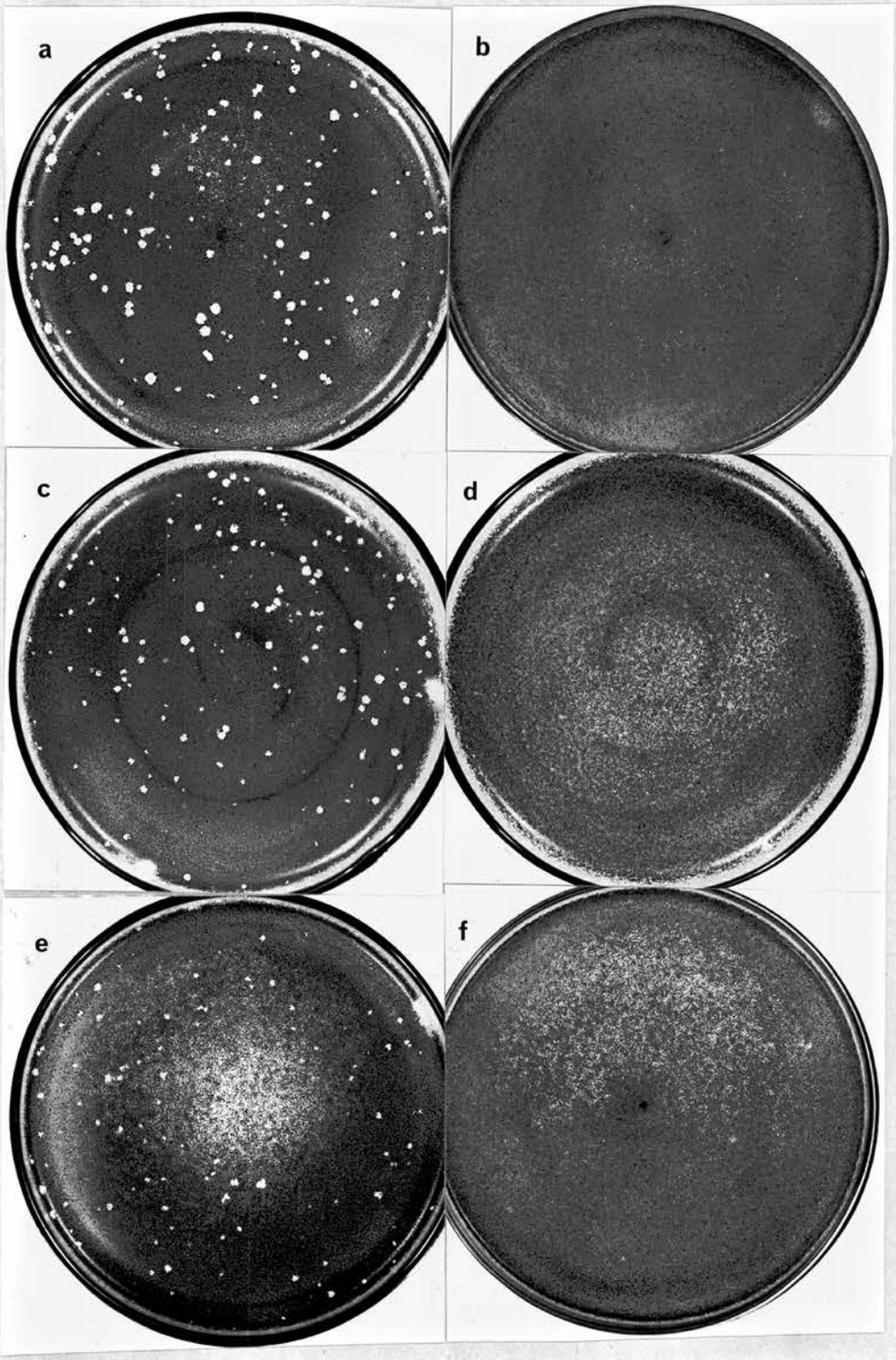
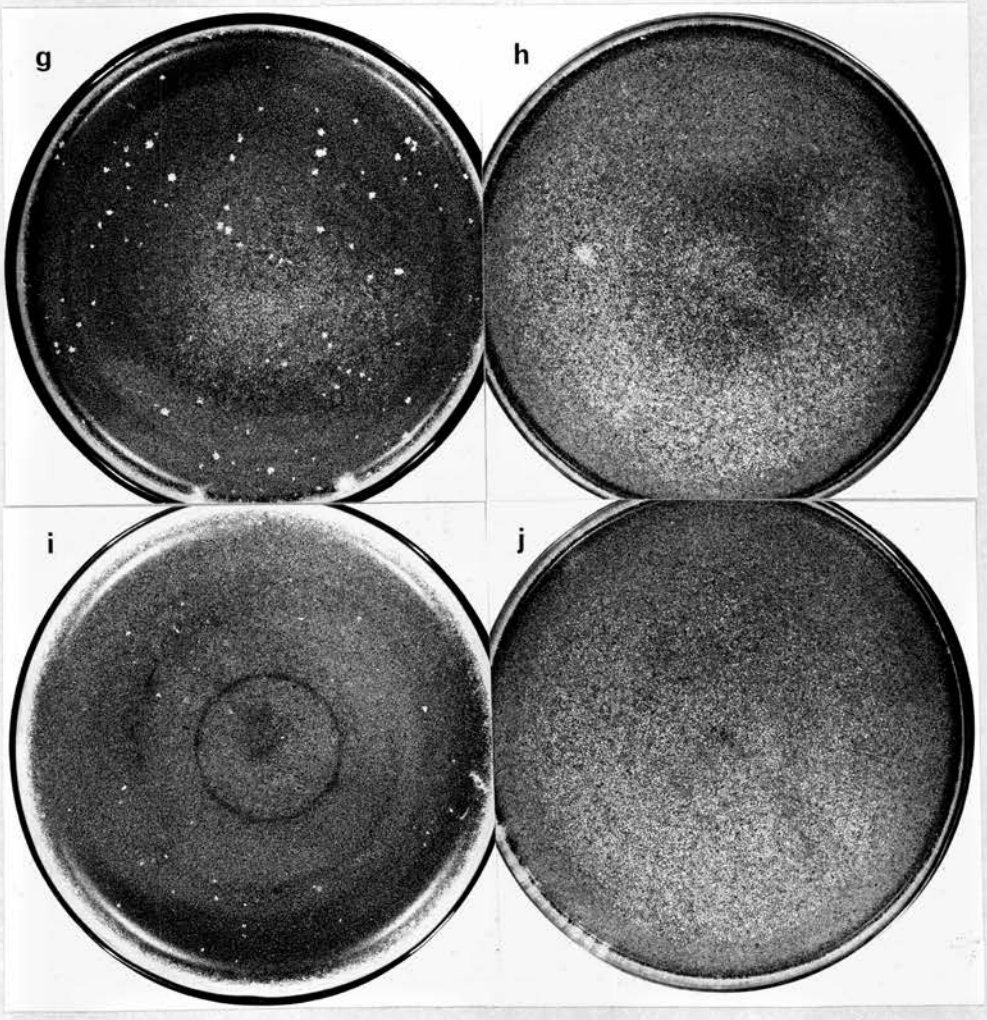


Figure iii (a - j)

The effect of varying concentrations of calf serum in Eagle's and in arginine-free medium on plaque formation by Herpes simplex virus (MP) in RK_{13} cells.



a and b: 20 per cent. calf serum Eag and Arg$^-$ respectively.
c and d: 10 " " " " " "
e and f: 5 " " " " " "
g and h: 1 " " " " " "
i and j: no " " " " " "

Petri dishes stained with 0.1 per cent. methyl violet.

Actual size.

after the 2 hours adsorption period and assayed to give some indication of the inoculum. The remainder were incubated at 37°C in Eag or Arg⁻ medium supplemented with 0-20 per cent. calf serum. Cultures were withdrawn at 12 hours and 24 hours post-infection, examined for viral CPE and assayed for virus yield.

The results obtained are shown in Figure iv(a-d).

Multiplicity of infection was calculated greater than 1 pl. f. u. per cell. When no calf serum was present in an Arg⁻ medium cultures infected at high multiplicities developed neither new infective virus nor viral CPE after 24 hours incubation at 37°C. With a 5 per cent. calf serum supplement Arg⁻ cultures showed no virus production after 24 hours, no viral CPE after 12 hours, but some small syncytia developed after 24 hours. The result with the 10 per cent. calf serum was similar except that the syncytia observed after 24 hours were extensive at the edges of the cell sheet. Cultures incubated in Arg⁻ medium supplemented with 20 per cent. calf serum produced new infective virus after 24 hours; traces of viral CPE were observed at 12 hours and these developed into large syncytia by 24 hours.

The corresponding results in the Eagle's medium are clearly expressed in Figure iv. It was seen in both the high and low multiplicity experiments that, in complete medium, the final virus yield increased as the concentration of calf serum increased.

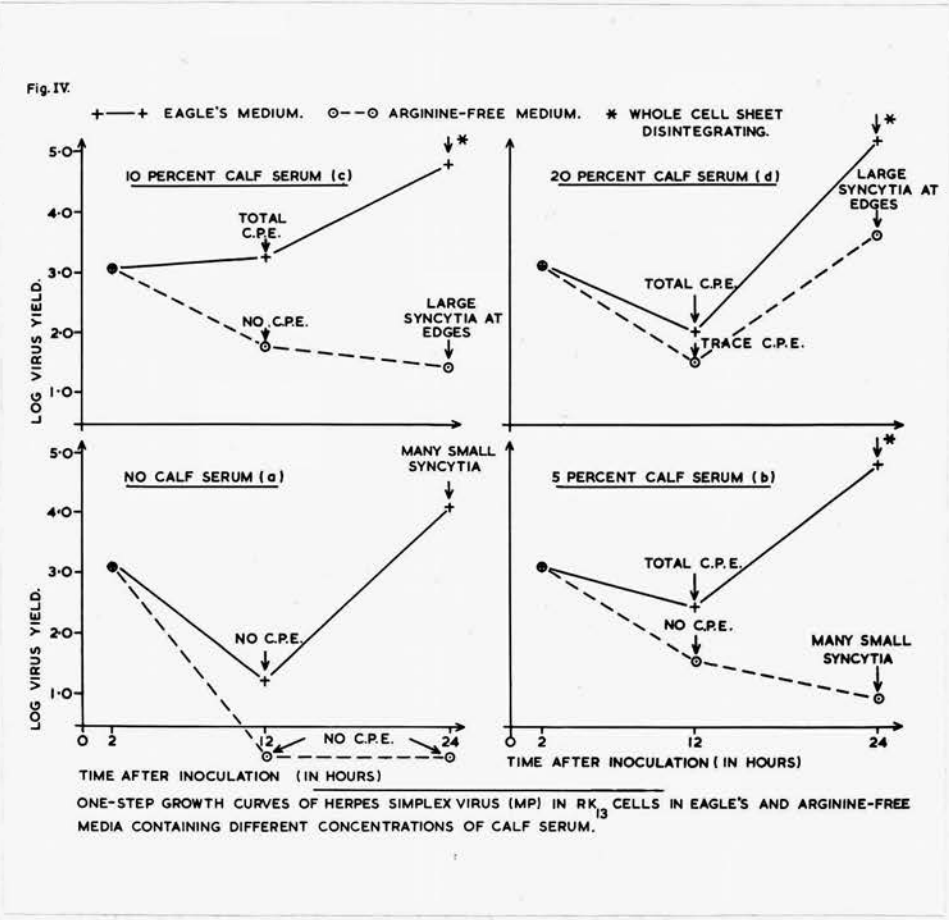


Figure iv. One-step growth curves in complete and deficient media which were supplemented with various concentrations of calf serum.

This figure shows the effect of calf serum concentration in normal medium on virus yield, and also the extent to which calf serum can substitute for arginine in a deficient medium.

In high multiplicity cultures in arginine deficient media, concentrations of calf serum greater than 5 per cent. resulted in widespread cytopathic effect. While this need not be associated with virus reproduction it eliminated the usefulness of these cultures as models of latent infections.

The growth of HSV(MP) in RK_{13} cells in a range of modified Eagle's media containing different concentrations of arginine was investigated. For this purpose the concentration of arginine in the standard Eagle's medium used here will be described as normal (N). Other concentrations will be expressed as multiples or fractions of this, e.g. 10. N, 5. N, $\frac{N}{2}$, etc.

Petri dish cultures were prepared, washed, inoculated and incubated in a range of media containing different concentrations of arginine. In those cultures to be examined for plaque formation media contained Methocel; in cultures to be assayed for virus yield there was no Methocel. Cultures were incubated for 60 hours at 37°C.

Results obtained are shown in Figure v.

Viral CPE developed in cultures containing $\frac{N}{2}$ or greater concentrations of arginine but this did not develop into plaques that could be counted with accuracy.

From these results there appeared to be a threshold level of arginine below which virus failed to grow.

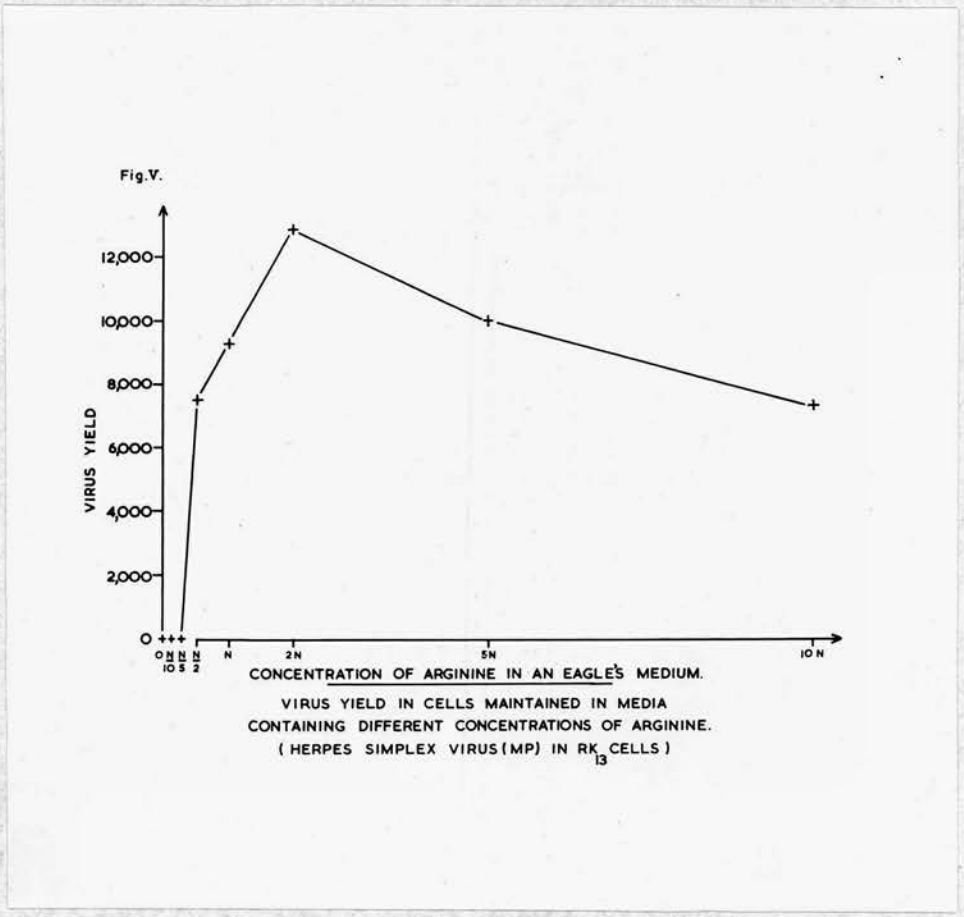


Figure v. To show the effect of arginine concentration in the medium on virus yield in HSV (MP) - infected RK_{13} cells.

Maximum virus yield was obtained with an arginine concentration of 2. N; excesses did not have further stimulatory effect on virus yield. The greatest concentration of arginine that could be added to the medium without supporting herpesvirus growth was $\frac{N}{5}$. Since it has already been observed that cells in serum-free Eagle's medium do not grow well, it was considered that little advantage would be gained in using an $\frac{N}{5}$ arginine-containing medium in preference to an arginine-free medium. The calf serum supplement approach appeared more promising.

v. Duration of Latency Induced by an Arginine Deficiency.

Based on the findings that no virus was produced in infected cells growing in 5 per cent. calf serum Arg^- medium and also that the infecting virus could be reactivated from the latent state by replacing arginine in the medium, the duration of survival of latent virus in a recoverable form was investigated.

A set of Carrel flask cultures of RK_{13} cells were prepared, washed and inoculated by the standard procedure. Three cultures received an overlay of 5 per cent. calf serum Eag medium and the remainder of 5 per cent. calf serum Arg^- medium. Cultures intended for estimation of plaque formation received Methocel overlays while cultures for assay of virus yield contained

no Methocel. After 48 hours incubation at 37°C, cultures were withdrawn and assayed to give the results shown in Table 12.

Table 12. Growth of HSV(MP) in RK_{13} cells in complete and in arginine deficient media.
Media contained 5 per cent. calf serum.

Medium	Number of plaques per petri dish	Number of pl.f.u. per petri dish
Eag	92	5.8×10^3
Arg^-	0	0

The remaining Arg^- cultures were withdrawn and assayed at intervals. After 5 days incubation one suppressed culture was subcultured into 3 Carrel flasks. Incubation in 5 per cent. calf serum Arg^- medium was continued and medium changed at approximately 4 day intervals. Cultures were withdrawn and assayed for infective virus between day 5 and day 15. On day 15 the medium on the one remaining culture was changed to 10 per cent. calf serum 199 and incubation continued.

The initial inoculum was 92 pl. f. u. per culture. Throughout this experiment there was no virus induced CPE nor production of infective virus in cultures maintained in 5 per cent. calf serum Arg^- medium. After subculture the cells did not grow well; a non-specific deterioration gradually developed with increasing

granularity and vacuolation of cells. At day 15 the remaining culture was in poor condition. However, after 7 days incubation in 10 per cent. calf serum 199 widespread syncytia were observed in the remaining culture. The syncytia developed from a number of foci suggesting that a considerable number of cells had been carrying latent virus rather than that CPE had gradually spread from one source.

These results suggested that in conditions of restricted arginine concentration cells remained viable for about two weeks and that throughout this time these cells were unable to support replication of herpesvirus. Moreover, it appeared that infected cells maintained in this deficient medium retained some 'memory' or 'blueprint' for virus reproduction for as long as the cells remained viable.

It had been found, however, that cells maintained with an arginine deficiency were difficult to subculture and so an experiment was designed to determine the survival of latent virus in cells without any attempt to subculture the cells.

Petri dish cultures were prepared, washed and inoculated as usual. Two cultures received 5 per cent. calf serum Eag medium and the remainder 5 per cent. calf serum Arg^- medium. All media contained Methocel. After 72 hours two Eagle's and two arginine deficient cultures were withdrawn and examined for plaque formation.

The Eagle's cultures showed about 700 plaques per petri dish, but the number was too high to be counted accurately. No plaques were seen on the Arg$^-$ cultures. At daily intervals in the beginning and less frequently later, pairs of arginine deficient cultures were withdrawn, examined microscopically for CPE and reincubated in 5 per cent. calf serum Eag medium to initiate recall of virus.

In this experiment the original inoculum was high since it was shown, see e.g. Table 5, that the number of foci that can be restimulated to form plaques decreases as the period of arginine starvation increases. The disadvantage was that this made it impossible to count plaque numbers accurately. A fair estimate was made, however, and this is recorded in Table 13.

It was observed microscopically from population density that some cells died and dropped off the glass during the experiment. For the first 7 days of suppression latent virus could be recalled and stimulated to form plaques that reached standard size in a further 2 days. Standard size in this experiment was that size achieved in cultures growing in 5 per cent. calf serum Eag medium after 3 days incubation. As the period of arginine deficiency extended beyond 7 days the time required for restimulation of virus growth by addition of complete medium increased. After 16 days suppression cells in the cultures appeared much smaller and thinner

Table 13. Duration of latency of HSV(MP) in RK_{13} cells induced by an arginine deficiency.

Duration of arginine deficiency (in days)	Number of plaques recalled per petri dish (approx.)	Time required for plaques to reach standard size (in days)
0	700	3
1	700	2
2	600	2
3	550	2
4	400	2
6	200	2
7	200	2
8	200	small at 2
10	100	" " 2
13	100	" " 3
16	100	6
21	100	*

* plaques developed without addition of complete medium

and the density of the cell population was considerably decreased. In these cultures plaques took longer to develop upon replacement of complete medium. After 21 days incubation in 5 per cent. calf serum Arg^- medium, syncytia developed without the addition of complete medium. It is significant that in these cultures areas of the cell sheet had completely disintegrated without any sign of viral CPE, in a manner similar to that seen in uninfected cultures grown in deficient media for long periods.

Throughout this experiment growth medium was changed every four or five days. It was unlikely that this should cause any secondary foci of infection since it had been found in earlier experiments that supernates from suppressed cultures contained no infective virus.

vi. The Intracellular Fate of a Latent Herpesvirus Particle.

Preceding experiments have shown that HSV(MP) in RK_{13} cells maintained in arginine deficient media was unable to undergo one complete replication cycle. An attempt was made to determine how far along the replication pathway the virus progressed before the cycle was stopped.

In dealing with a nutrient deficiency of this type there was a distinct possibility that results would be considerably influenced by the immediately previous

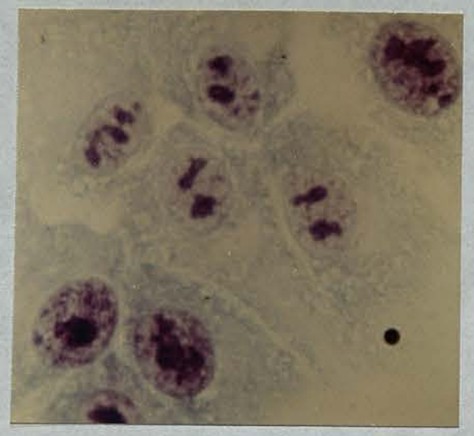
nutritional history of the culture. A prolonged starvation period prior to inoculation, for example, might affect where the breakdown in viral synthesis occurred. For this reason all experiments whether extending over one replication cycle or over several days started from cultures as like as possible. This has been described in detail before, but can be summarised usefully here.

Experimental cultures were prepared from 6 day old stock cell cultures by inoculation with a constant number of cells according to the vessel used. Cells were always sown in a constant volume of 5 per cent. calf serum 199 and were incubated overnight at 37°C to form monolayers. The subsequent washing, inoculating, and further washing has already been described. Unless specifically stated there was no period of arginine starvation prior to inoculation with virus.

It has already been observed that in arginine-free medium herpesvirus adsorbs, penetrates and infectivity is eclipsed at the normal rate.

The development of viral inclusions, in particular the Cowdray type A nuclear inclusions characteristic of herpesvirus, was investigated by Giemsa staining of infected cultures. Cover slip cultures were prepared and inoculated in the usual manner and then incubated in Eag or Arg^- medium. Specimens were withdrawn after 24

a



b

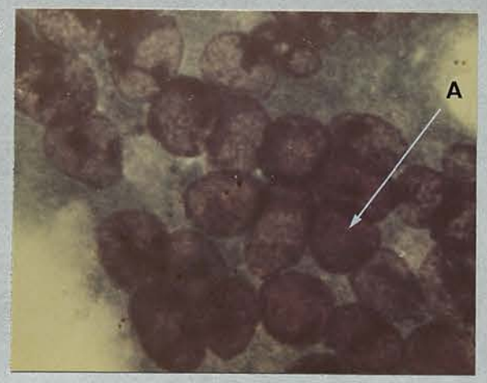
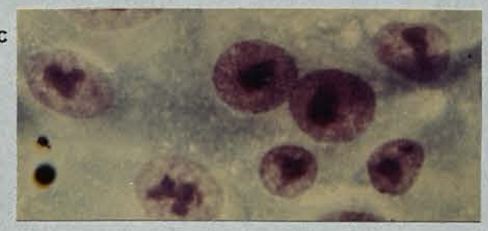


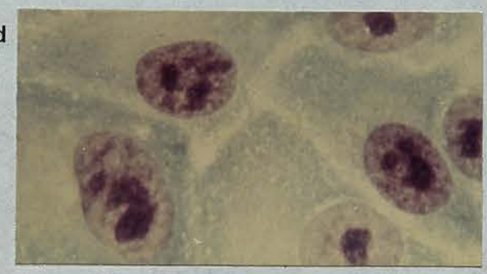
Figure vi. Giemsa staining to show cytopathic changes induced by Herpes simplex virus (MP) in RK_{13} cells after incubation for 24 hours in Eagle's or in arginine-free medium.

a) Uninfected cells in Eagle's medium.
b) RK_{13} cells, infected with HSV(MP), showing characteristic syncytial formation.
A Cowdray type A intranuclear inclusion (A) is indicated.

c



d



c) Uninfected cells incubated in arginine-free medium.
d) RK_{13} cells infected with HSV(MP) and incubated in arginine-free medium. Under these conditions no cytopathic changes were observed.

Cultures were stained overnight with Giemsa's stain and differentiated by immersing for 10 seconds in colophonium in methanol.

x 1,000

hours, stained by Giemsa's method and examined. Uninfected controls were similarly examined. See Figure vi (a-d).

In HSV(MP)-infected cells which had been incubated in Eag medium, syncytial formation was observed after 24 hours incubation. Eosinophilic intranuclear bodies were observed in some of these infected cells. No cytopathogenic changes were observed in infected cells which had been incubated in Arg^- medium or in the uninfected control cultures which had been incubated in Eag and Arg^- media respectively.

The formation of virus induced proteins in infected cells growing in an arginine-free medium was investigated by testing for complement fixing antigens and also by the fluorescent antibody staining technique.

Complement fixation tests were carried out on water-lysed samples of infected cells. The samples were prepared in a previously described experiment, see Figure ii. Samples were tested after 0, 2, 6 and 23 hours incubation in Eag and Arg^- media. Uninfected controls which had been incubated for 23 hours in Eag or Arg^- media were included. The results obtained are expressed in Table 14 as reciprocals of the complement fixing titre. In this test a one tube difference is not considered significant.

Table 14. Complement-fixing antigen produced in HSV(MP)-infected RK_{13} cells growing in an Eagle's and in an arginine-free medium.

Time post-inoculation at which sample was withdrawn (in hours)	Titre, expressed as a reciprocal, of complement-fixing antigen from infected cells grown in:	
	Eag	Arg^-
0	8	8
2	16	8
6	8	16
23	64	8
Uninfected controls	2	2

The results in Table 14 indicate the production of a complement-fixing antigen in HSV(MP)-infected RK_{13} cells after 23 hours incubation in Eag medium, but no corresponding antigen production in cells incubated in Arg^- medium.

Cover slip cultures were prepared and inoculated for the fluorescent antibody staining test. Samples were withdrawn and stained immediately after the adsorption period, after 4½ hours and after 11 hours incubation in Eag and Arg^- media. Uninfected cell controls which had been incubated in the two media were also stained after 11 hours. Cultures were examined under ultra-violet illumination.

In all cultures there was a slight background of

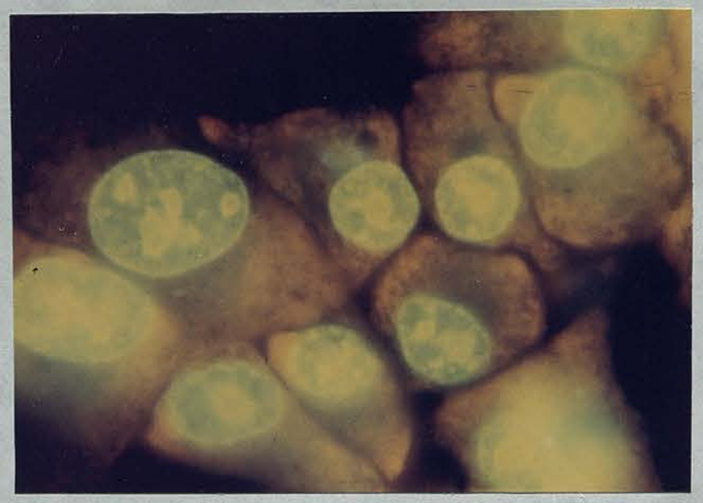
pale green fluorescence. The 2 hour samples, i.e. those taken after adsorption period, showed spots of bright green fluorescence in the cytoplasm of the cells. Only background fluorescence was observed in the 4½ hour samples. In the 11 hour Eag samples some small syncytia had formed and they fluoresced brightly. There was no corresponding fluorescence in the 11 hour Arg^- samples. The uninfected controls showed only non-specific background staining.

This is a very delicate test and requires considerable experience before it becomes a reliable tool. A comparatively small number of tests were carried out here and this was probably insufficient to permit placing a high degree of confidence in these findings. These results will be taken, therefore, only as an indication that there is either a very limited amount or no synthesis of virus specific protein in infected cells growing in an arginine-free medium.

An investigation of virus-induced nuclear changes in infected cells growing in complete and in arginine deficient medium was made by acridine orange staining.

Cover slip cultures were prepared, infected and incubated in Eag or Arg^- medium. Samples were withdrawn for up to 24 hours after inoculation and stained with acridine orange. Uninfected cell controls were included for both media and stained similarly. Specimens

a



b

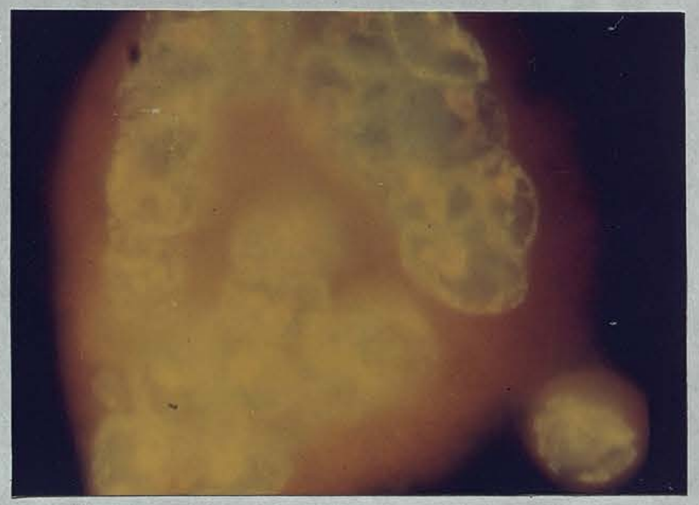


Figure vii (a - d)

Acridine orange staining to show nuclear changes induced by Herpes simplex virus (MP) in RK_{13} cells after incubation for 24 hours in Eagle's or in arginine-free medium.

a) Uninfected RK_{13} cells in Eagle's medium.

b) RK_{13} cells, infected with HSV (MP), in Eagle's medium showing characteristic nuclear changes. Margination of the chromatin around the periphery of the nucleus; tendency to disappearance of the nucleoli; and aggregation of nuclei inside syncytial masses.

c

d

c) Uninfected RK_{13} cells incubated in arginine-free medium.

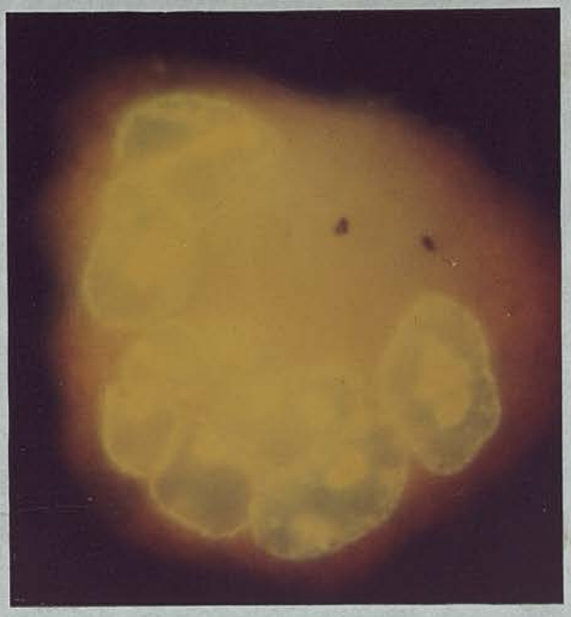
d) RK_{13} cells infected with HSV (MP) and incubated in arginine-free medium.

Under these conditions no nuclear changes were observed.

Cultures stained with a 0.1 per cent. solution of acridine orange in buffer at pH 3.8

x 930

e



f

Figure vii continued (e and f)

Acridine orange staining to show virus-induced nuclear changes.

e) RK_{13} cells, infected with HSV (MP) and incubated in Eagle's medium, showing the normal type of virus-induced syncytium.

f) RK_{13} cells, infected with HSV (MP) and incubated in arginine-free medium showing a small syncytium. Such cytopathic effect was very rarely observed in these cultures.

x 930

were examined under ultra-violet illumination and typical fields were recorded photographically. These are shown in Figure vii (a-f).

Uninfected cells from Eag and Arg^- media appeared similar. The RNA of the cytoplasm fluoresced red, the DNA of the nucleus fluoresced green and within the nucleus the nucleoli appeared yellow-orange. This also was the appearance of the majority of infected cells that had been incubated for 24 hours in Arg^- medium. A very small proportion, perhaps one or two groups of cells per cover clip, gathered to form small syncytia as is demonstrated in Figure vii (f). In infected cells which had been incubated for 24 hours in Eag medium characteristic nuclear changes occurred. There was a gradual disappearance of the nucleoli and banding of chromatin around the periphery of the nucleus. This started about 8 hours post-infection. Nuclei aggregated together inside syncytial masses of cytoplasm. These changes were complete by 24 hours post-infection.

Electron microscopic studies of HSV(MP)-infected RK_{13} cells growing in arginine deficient medium were made to investigate the possible development of incomplete or non-infectious virus particles under these nutritional conditions. Virus particles were adsorbed to RK_{13} cells at $4^{o}C$ as described for the experiment illustrated by Figure ii. The fate of these particles when the cells

were incubated in Eag and Arg^- media was followed by electron microscopy of ultra-thin section of cells.

Withdrawal of samples, fixation and preparation for electron microscopy has already been described. As stated, the samples selected for thin-sectioning were paralleled by the assay of infectivity as in Figure ii and the results of the complement fixation test given in Table 14.

There follows a series of representative electron micrographs illustrating the events occurring in HSV(MP)-infected RK_{13} cells when these cells were grown in Eag and in Arg^- media.

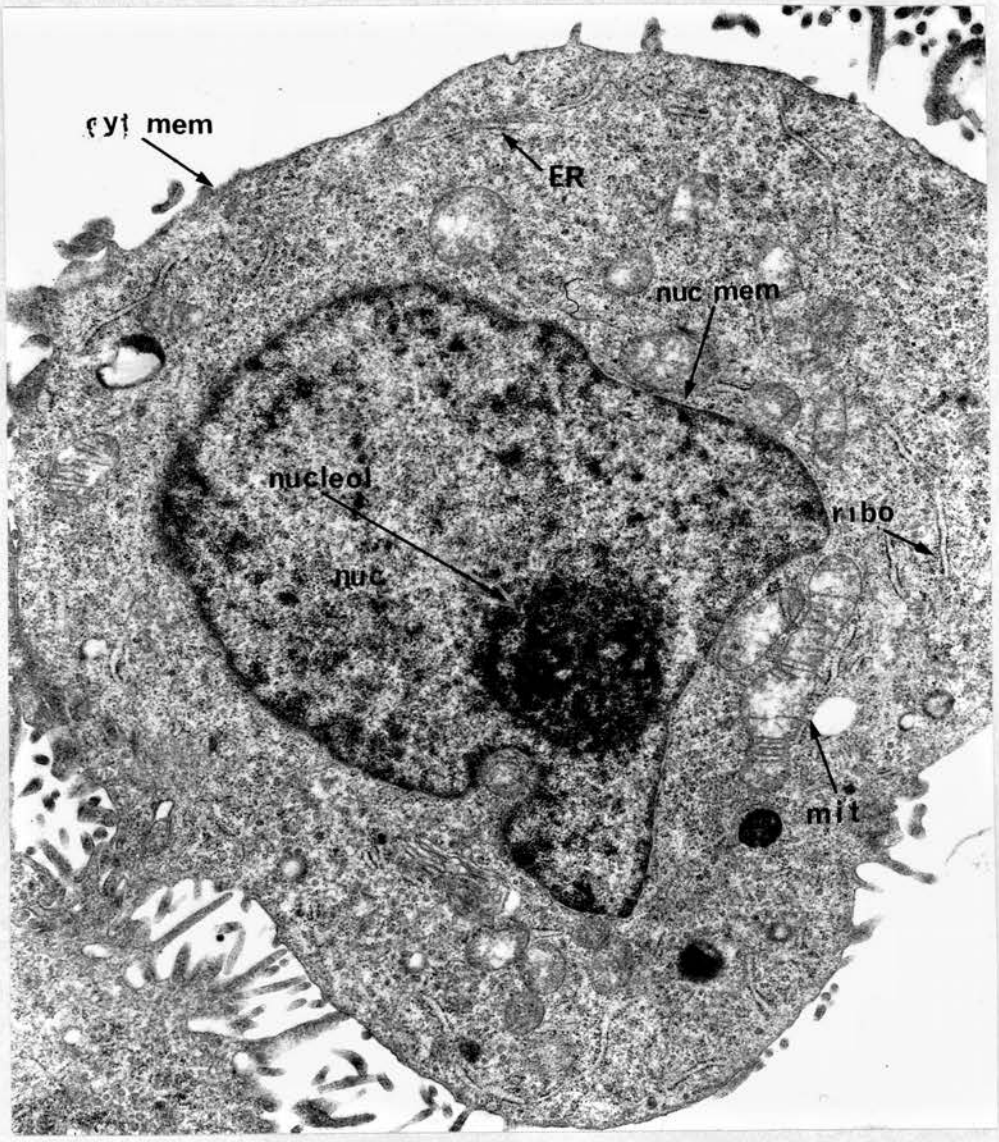


Figure viii (a)

Eagle's medium. Uninfected RK_{13} cell showing normal cytology.

The following can be seen:

nucleus (nuc)
nuclear membrane (nuc mem)
nucleolus (nucleol)
endoplasmic reticulum (ER)
mitochondria with christae (mit)
ribosomes (ribo)
cytoplasmic membrane (cyt mem)

x 12,000

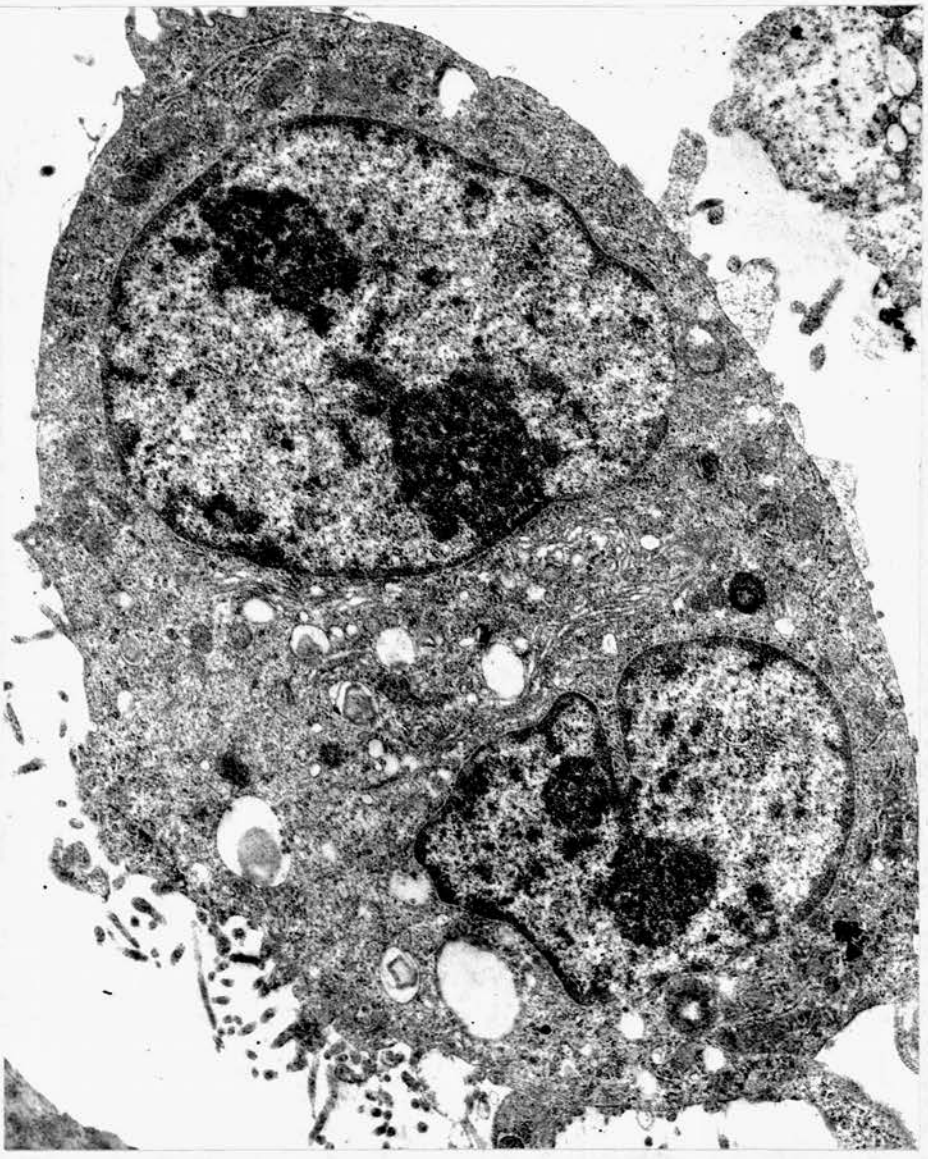


Figure viii (b)

Eagle's medium. Uninfected RK_{13} cell in which the nucleus had divided but the cytoplasm had not yet separated to form two cells.

This is included for comparison with a virus-induced syncytium; see fig. viii (k).

x 12,000

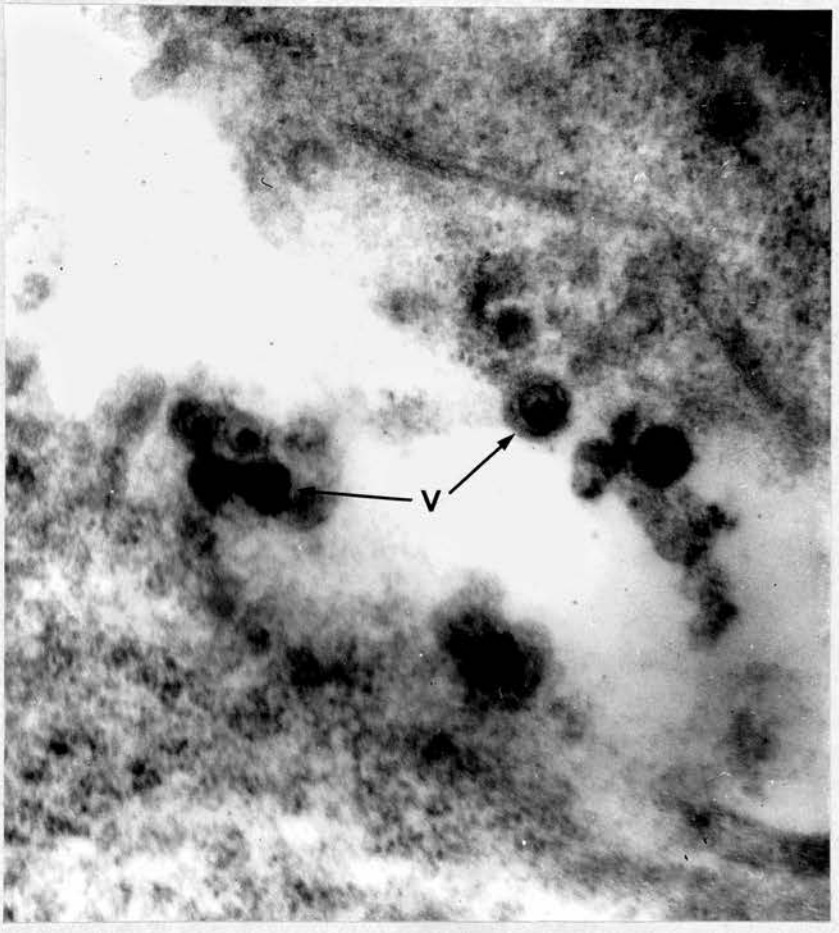


Figure viii (c)

<u>0 hours post-inoculation</u>.

RK_{13} cells showing Herpes simplex virus particles (V) which had adsorbed but had not begun penetration.

x 60,000

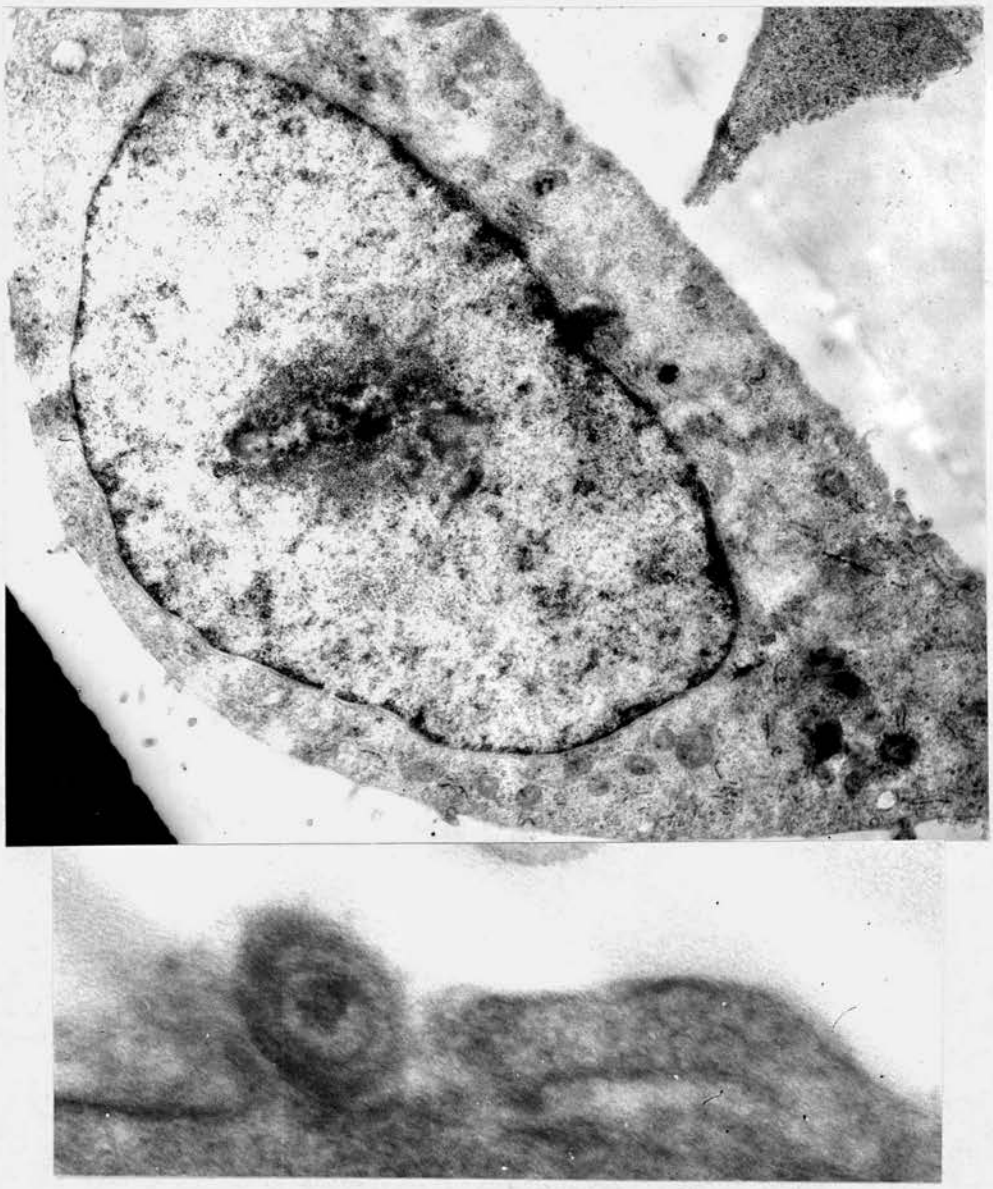


Figure viii (d)

Eagle's medium: 1 hour post-inoculation.

No attached virus particles were seen in this section although all cells in the population had been inoculated. The nucleus remained unchanged. The inset shows a virus particle adsorbed to the cytoplasmic membrane of another cell in this sample.

x 12,000
x 150,000

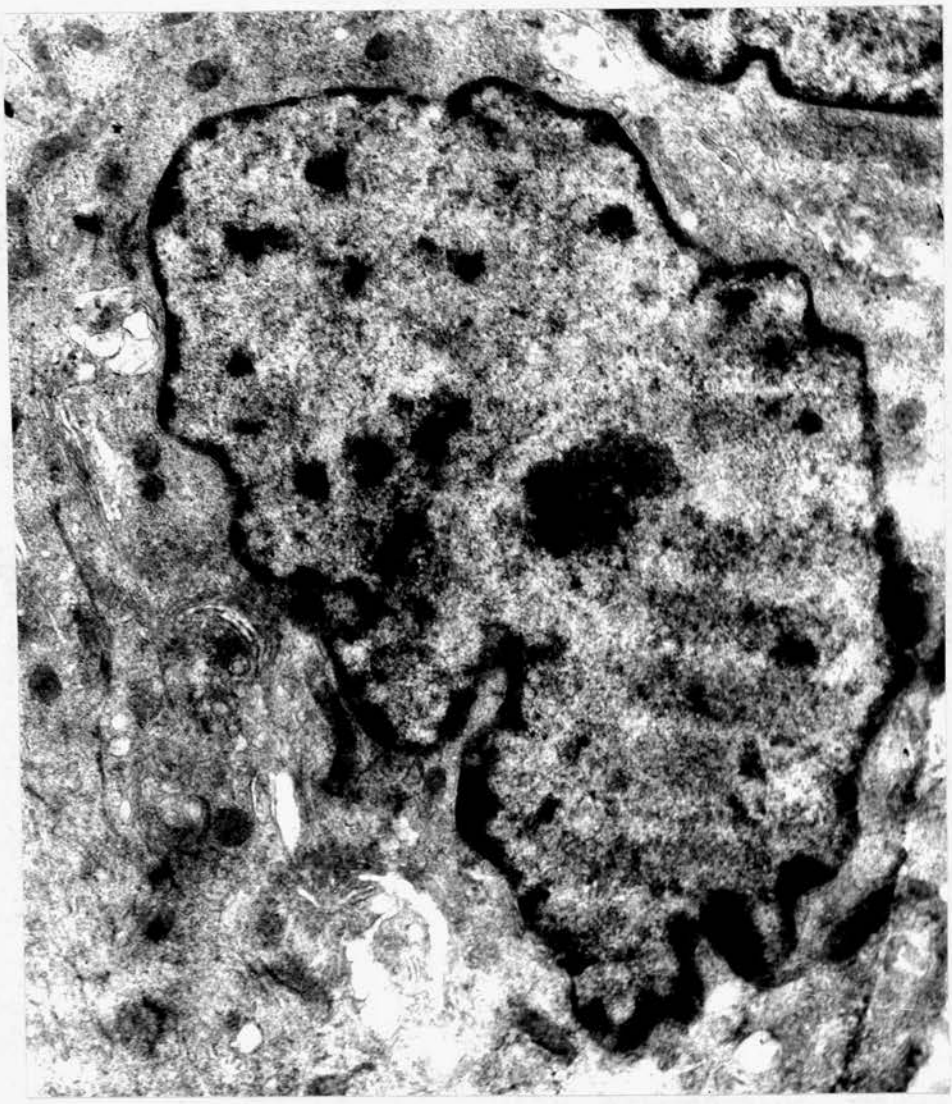


Figure viii (e)

Eagle's medium: 2 hours post-inoculation.

No virus particles were seen either adsorbed to the cytoplasmic membrane or in the cytoplasm of these cells. The nuclei retained the normal appearance and the nucleoli remained intact.

x 16,000

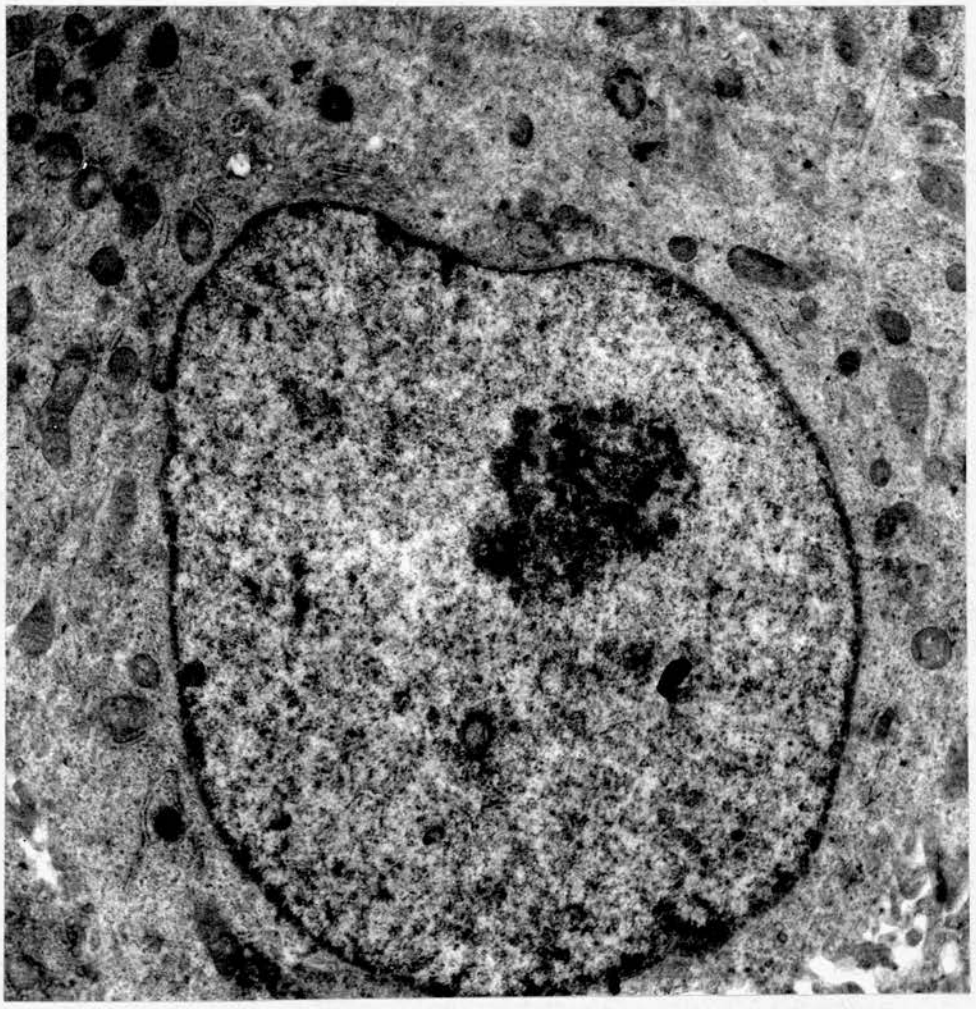


Figure viii (f)

Eagle's medium: 3 hours post-inoculation.

No virus particles were observed in these cells. No nuclear or cytoplasmic changes were seen.

x 12,000

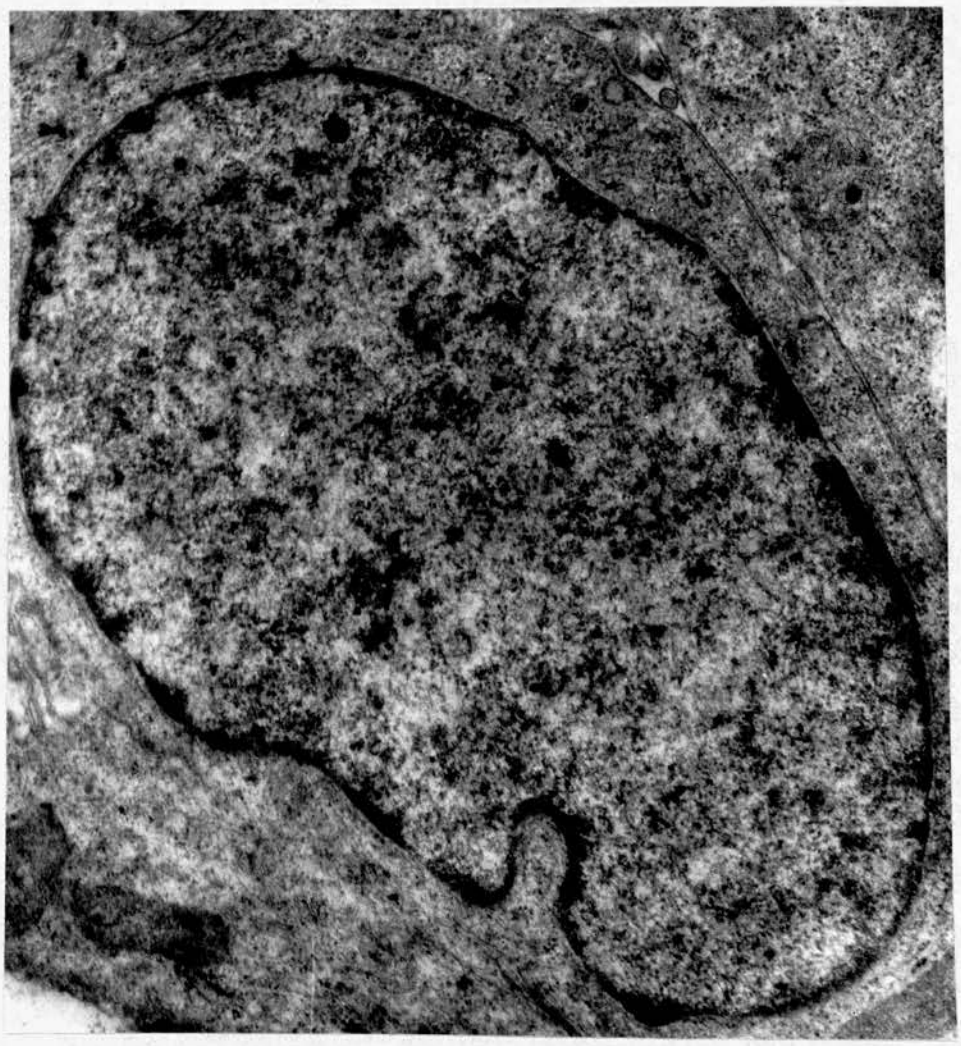


Figure viii (g)

Eagle's medium: 6 hours post-inoculation.

No virus particles were observed. Nucleoli are absent in this section, however this alone is not indicative of virus growth as presence or absence of nucleoli in a section depends on where the cell was cut. No definite virus-induced changes were seen.

x 32,000

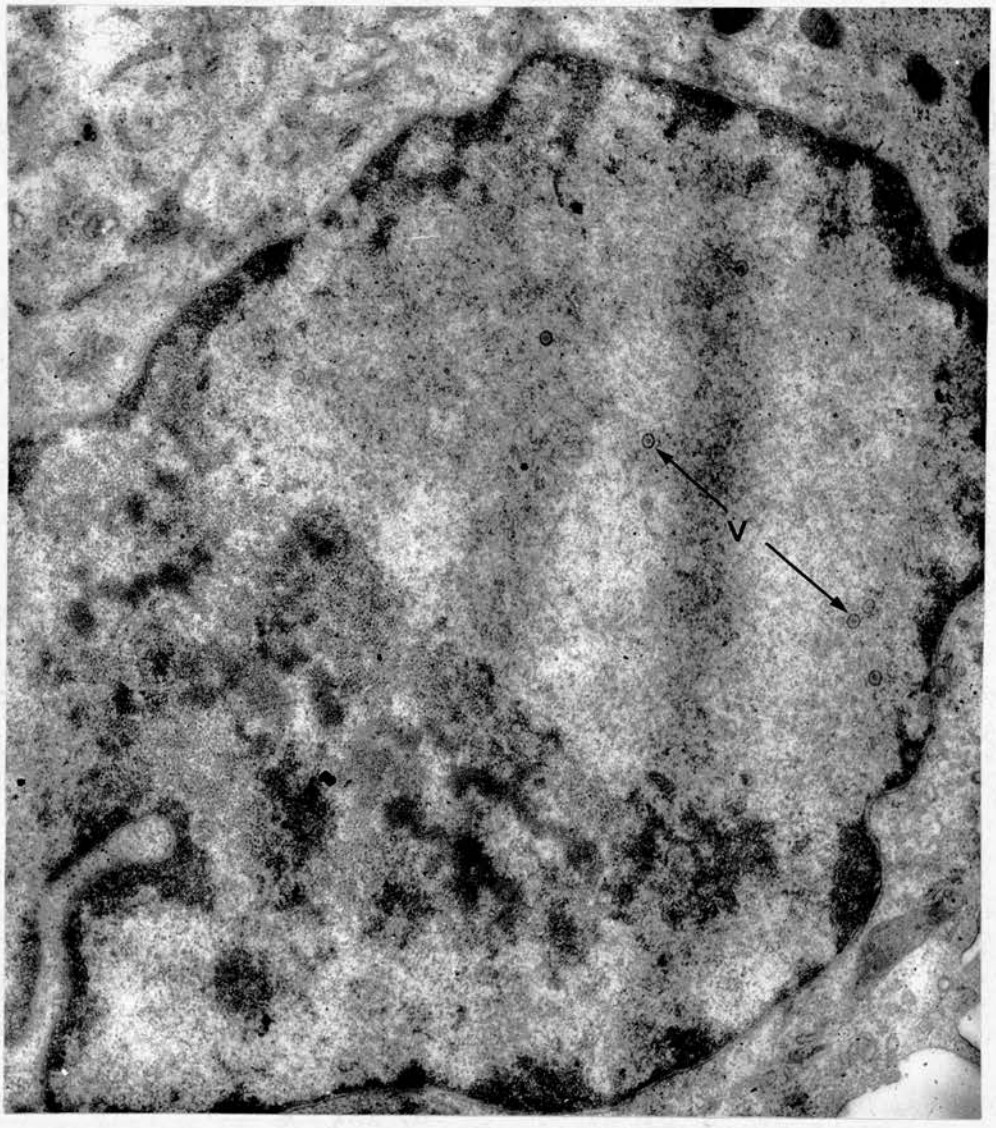


Figure viii (h)

<u>Eagle's medium: 9 hours post-inoculation</u>.

This section illustrates a tendency to disappearance of the nucleoli and margination of the chromatin. Virus particles (V) can be seen in the nucleus but these were randomly distributed. No cytoplasmic changes were seen.

x 20,000

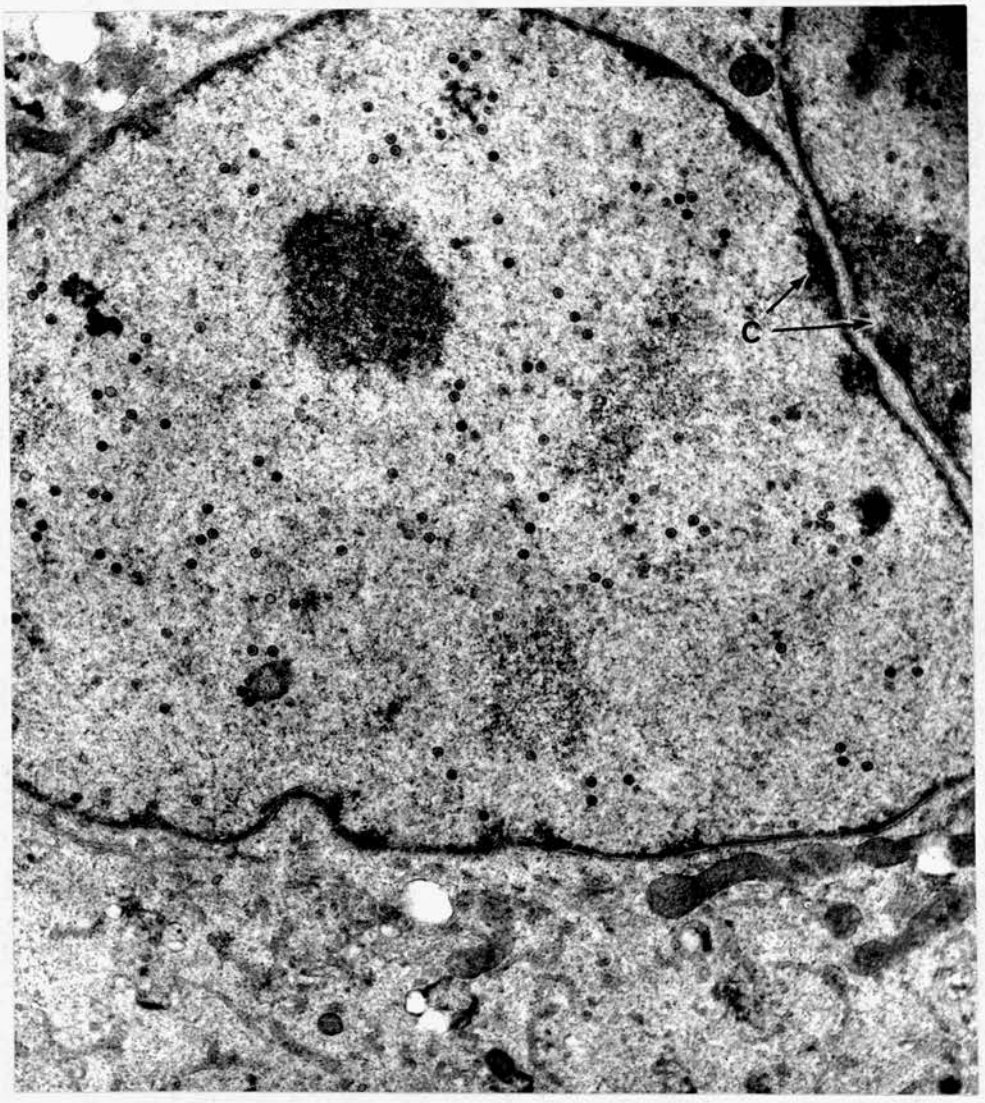


Figure viii (i)

Eagle's medium: 11 hours post-inoculation.

This shows parts of two nuclei in a virus-induced syncytium. Many virus particles can be seen in the nuclei; these were randomly distributed. There was distinct margination of the chromatin (C).

x 16,000

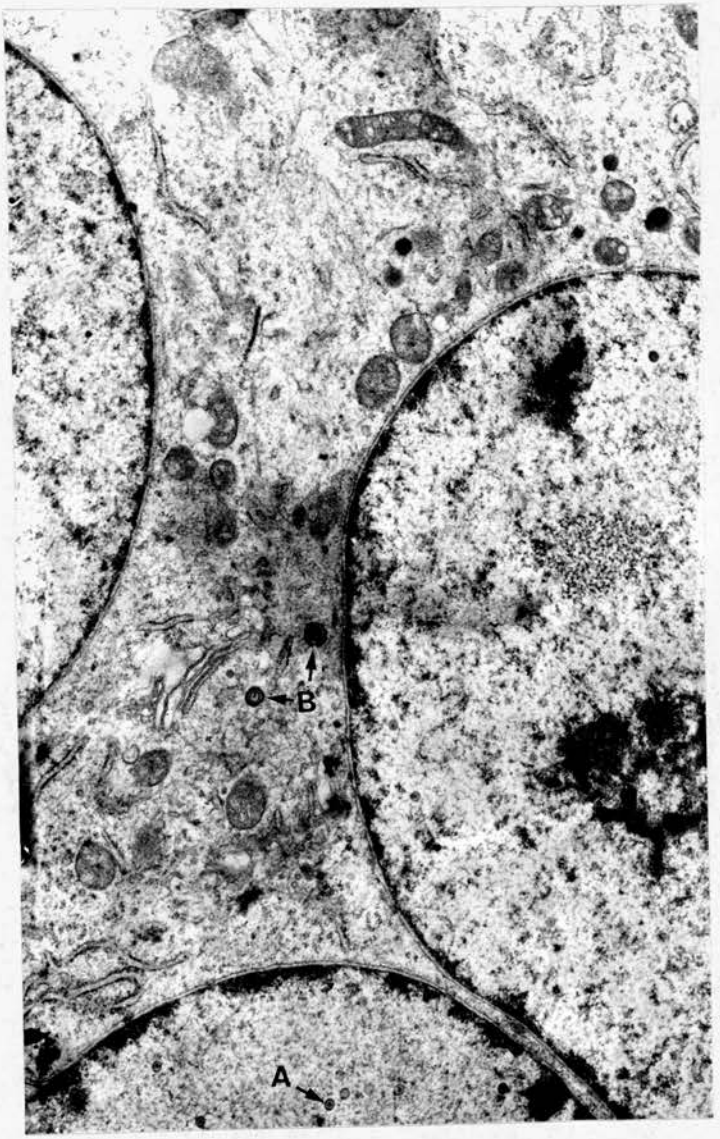


Figure viii (j)

Eagle's medium: 11 hours post-inoculation.

This section shows parts of three nuclei in a virus-induced syncytium. Naked virus (e.g. A) can be seen in the nuclei. In the cytoplasm there are virus particles (e.g. B) inside an envelope derived from the nuclear membrane. The cytoplasm of the infected cells still contains mitochondria, endoplasmic reticula and other unaltered structures.

x 16,000

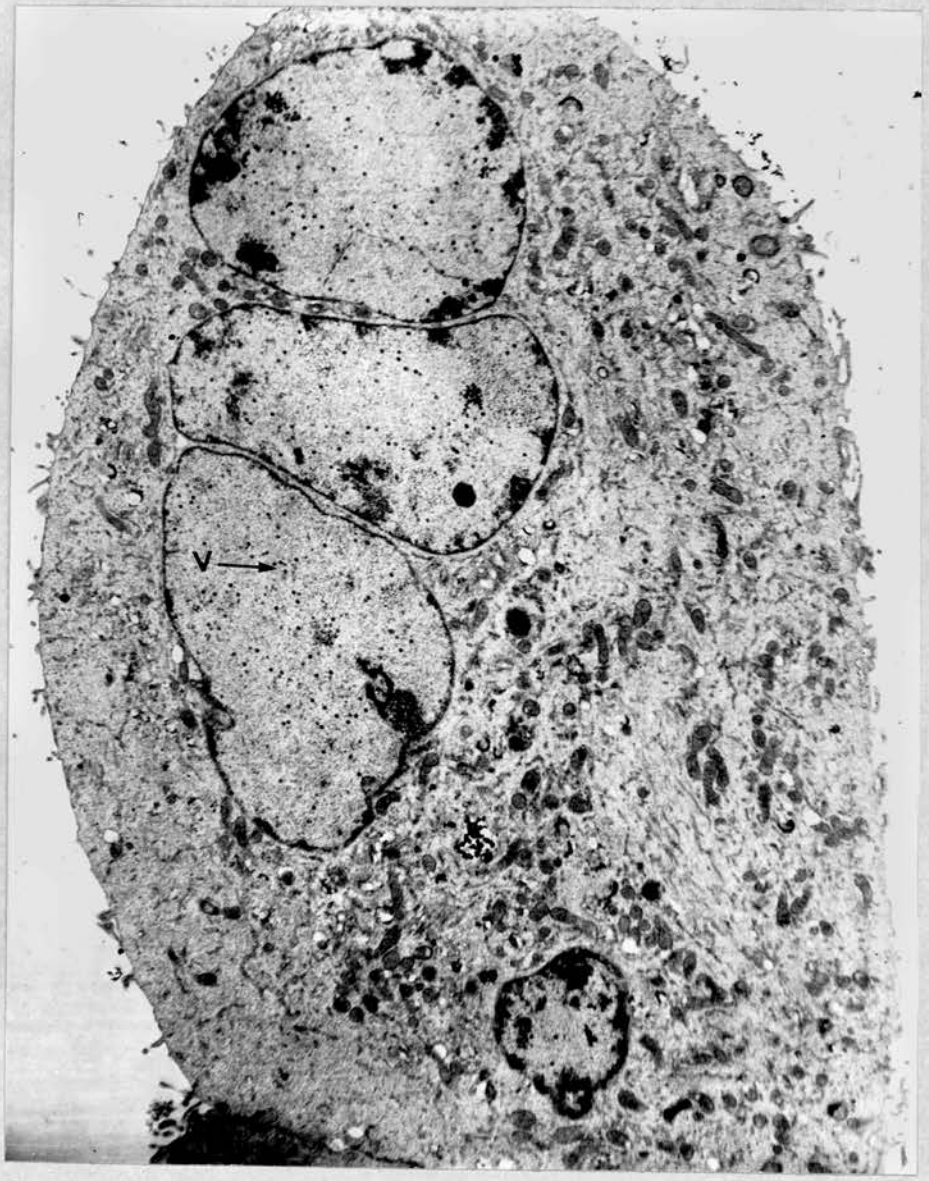


Figure viii (k)

Eagle's medium: 23 hours post-inoculation.

This section shows a virus-induced syncytium with four nuclei each containing virus particles. The disappearance of the nucleoli and margination of the chromatin has occurred in all four nuclei. Other structural components in the cytoplasm remained clearly identifiable. Compare this with fig. viii (b), an uninfected cell in which the nucleus has just divided.

x 3,000

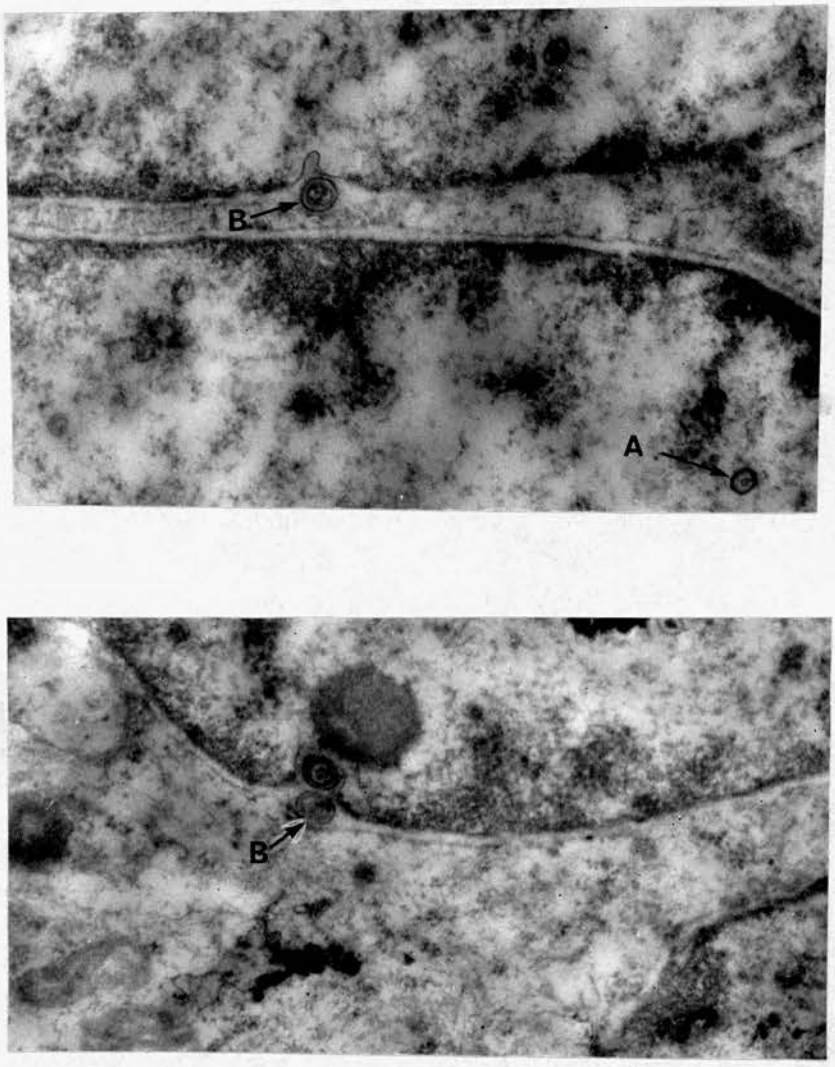


Figure viii (1)

Eagle's medium: 23 hours post-inoculation.

This shows virus particles passing out of the nucleus into the cytoplasm. Particles which have left the nucleus are surrounded by an extra membrane (B) as compared with particles still inside the nucleus (A).

x 40,000

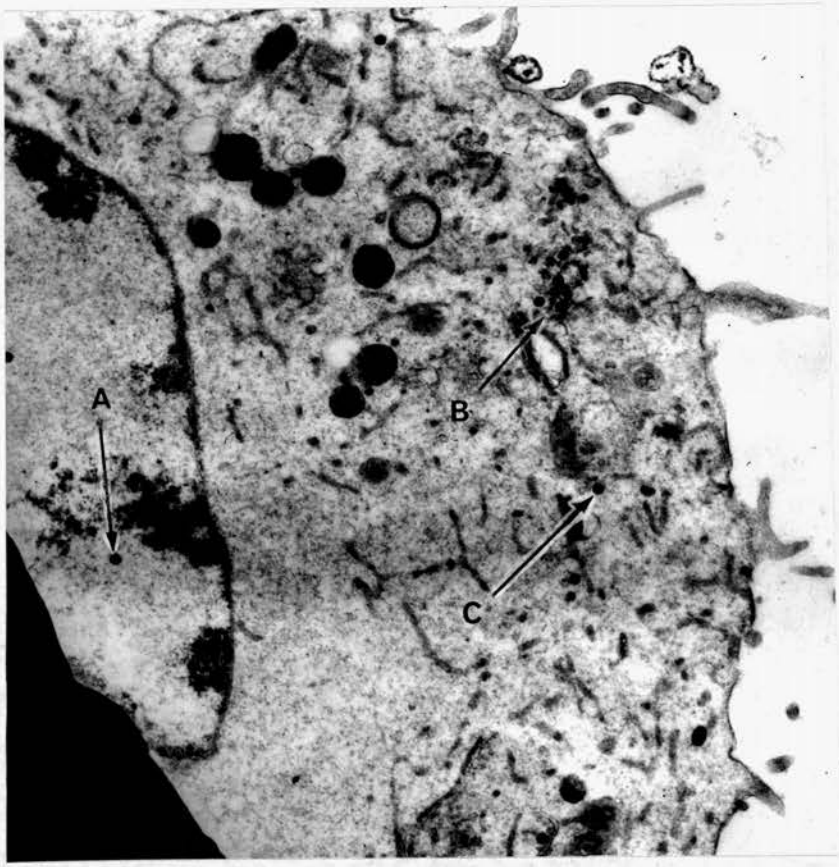


Figure viii (m)

Eagle's medium: 23 hours post-inoculation.

In this cell virus particles can be seen in the nucleus (A) and others in the cytoplasm either in groups (B) or singly (C) and apparently contained in a large enveloping membrane.

x 16,000

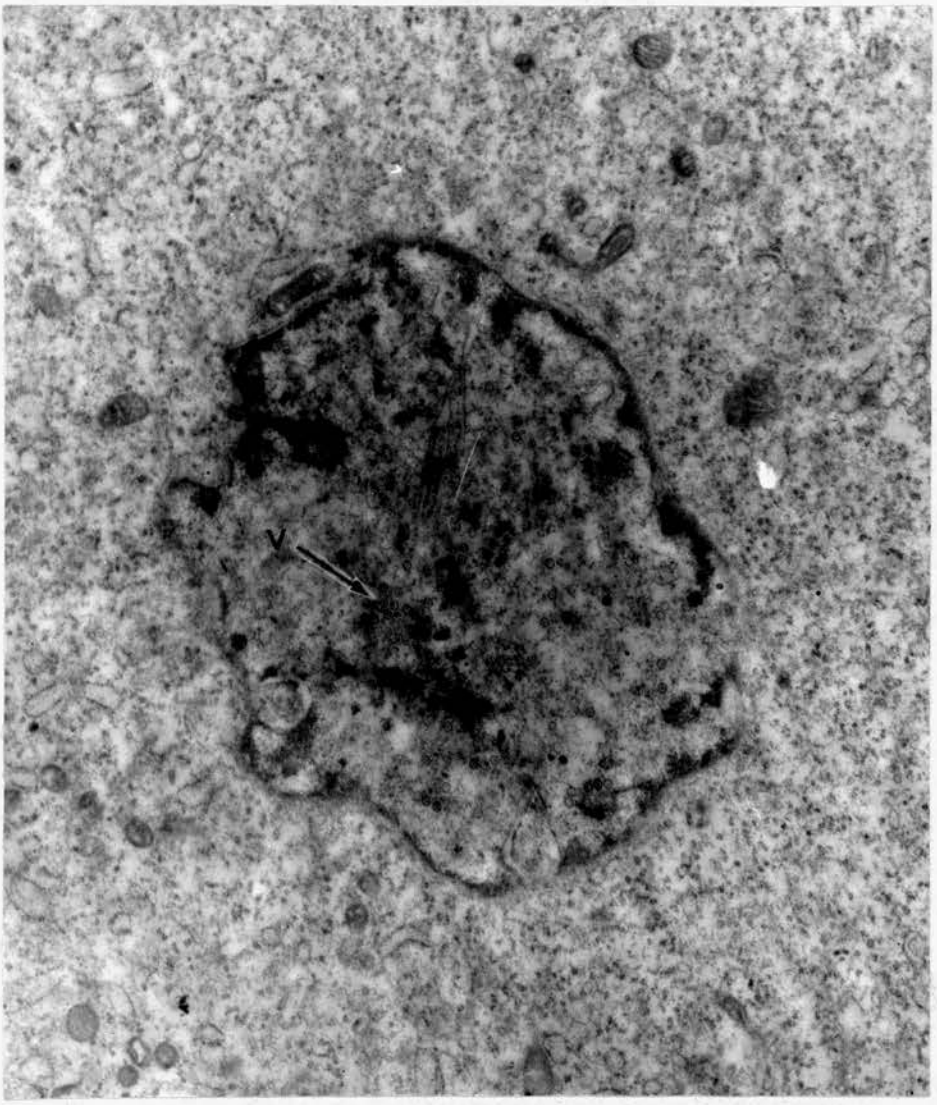


Figure viii (n)

Eagle's medium: 23 hours post-inoculation.

This section shows the nucleus of an RK_{13} cell containing many virus particles, organised in something approaching crystalline array.

x 16,000

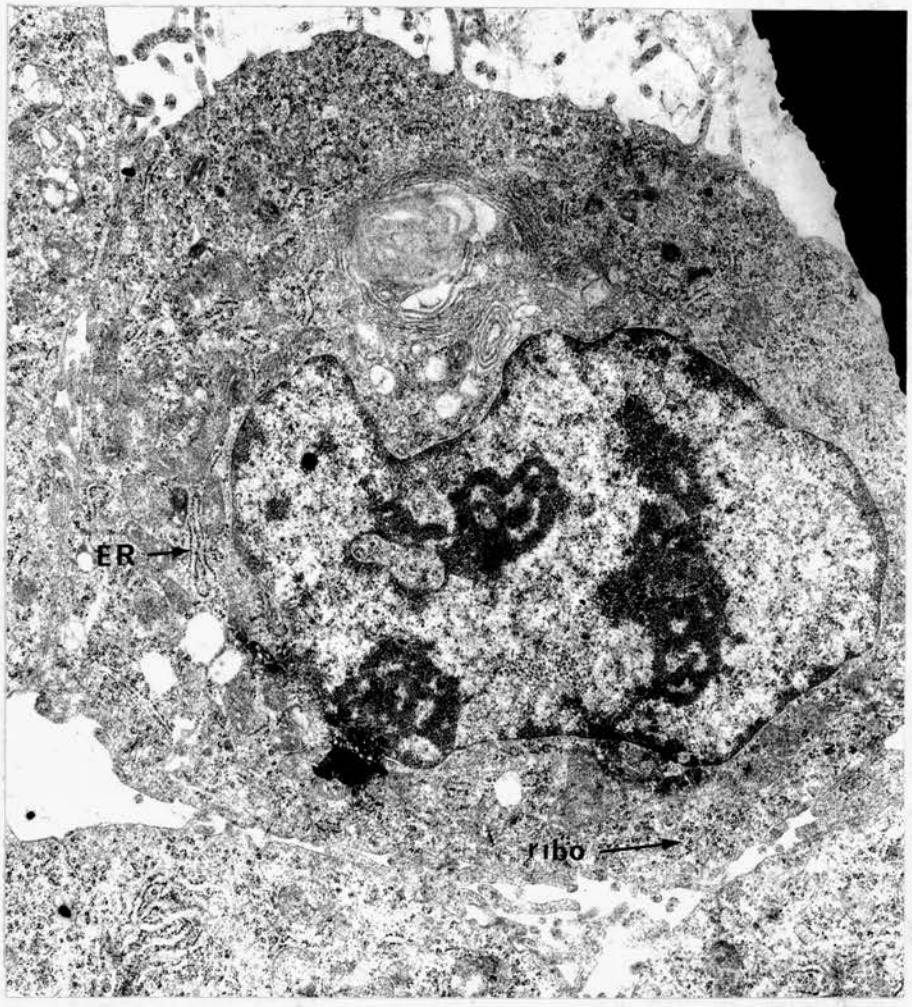


Figure viii (o)

Arginine-free medium.

An uninfected RK_{13} cell, which had been incubated for 23 hours in arginine-free medium, still shows normal cytology. Compare with fig. viii (a). In particular, the endoplasmic reticulum (ER) and ribosomes (ribo) are clearly seen.

x 12,000

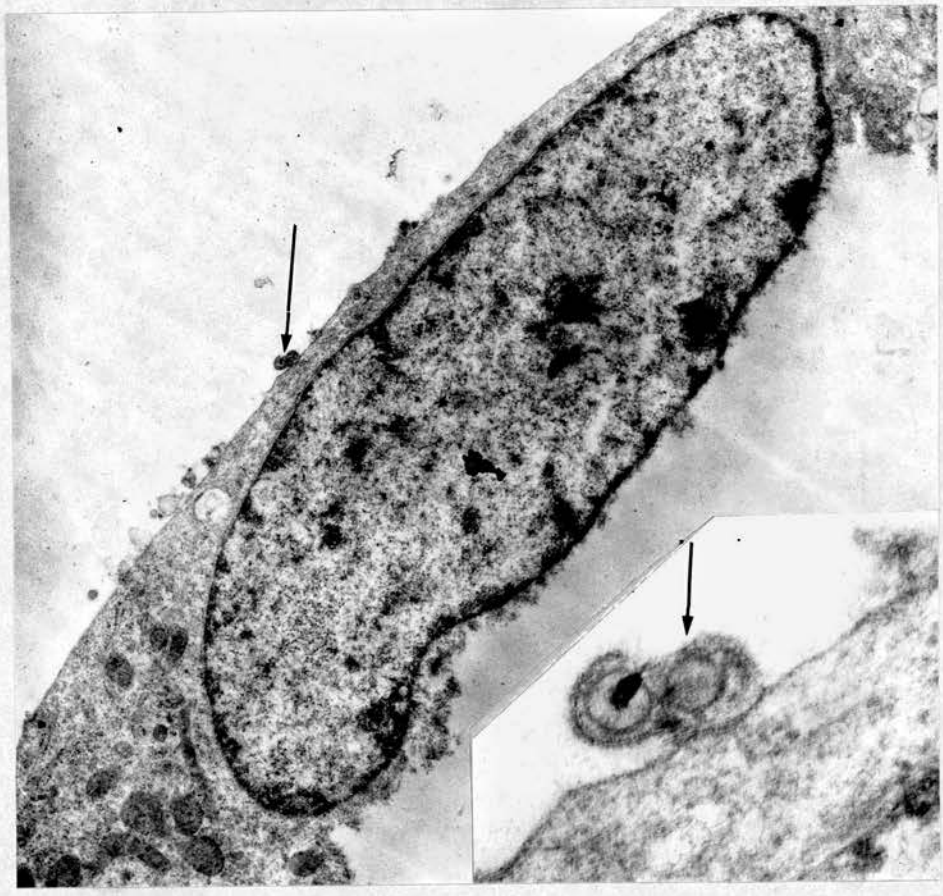


Figure viii (p)

Arginine-free medium: 1 hour post-inoculation.

The 0 hour post-inoculation sample, fig. viii (c), is common to both series. The 1 hour sample shows virus adsorbed to the outside of a cell; the inset is an enlargement of this. In these cells there was no change in the nuclei or cytoplasm of infected cells.

x 12,000
x 100,000

Figure viii (q)

Arginine-free medium: 2 hours post-inoculation.

No virus particles were observed either adsorbed to the cytoplasmic membrane or penetrated into the cytoplasm. No nuclear changes were observed.

x 16,000

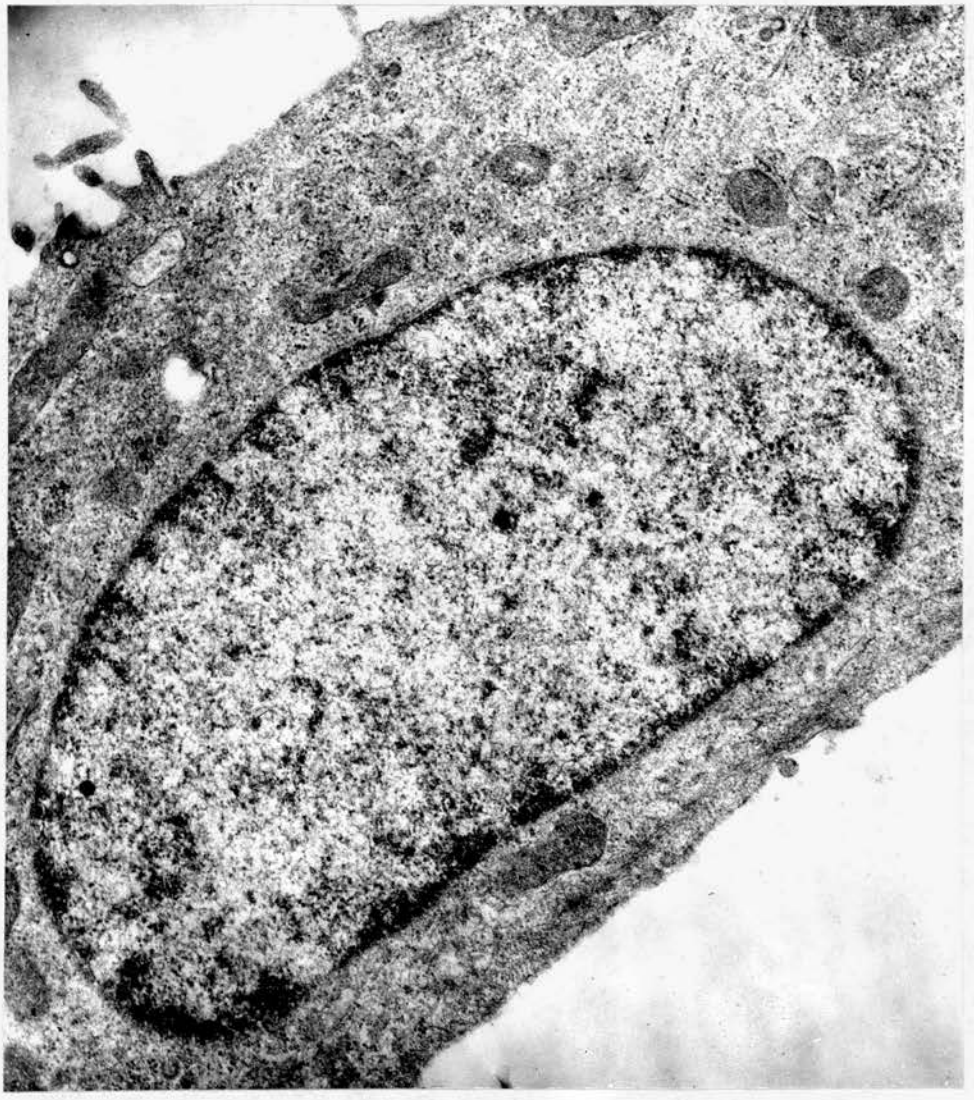


Figure viii (r)

Arginine-free medium: 3 hours post-inoculation.

Nothing was observed which could be identified as virus particles or viral inclusions.

x 26,000

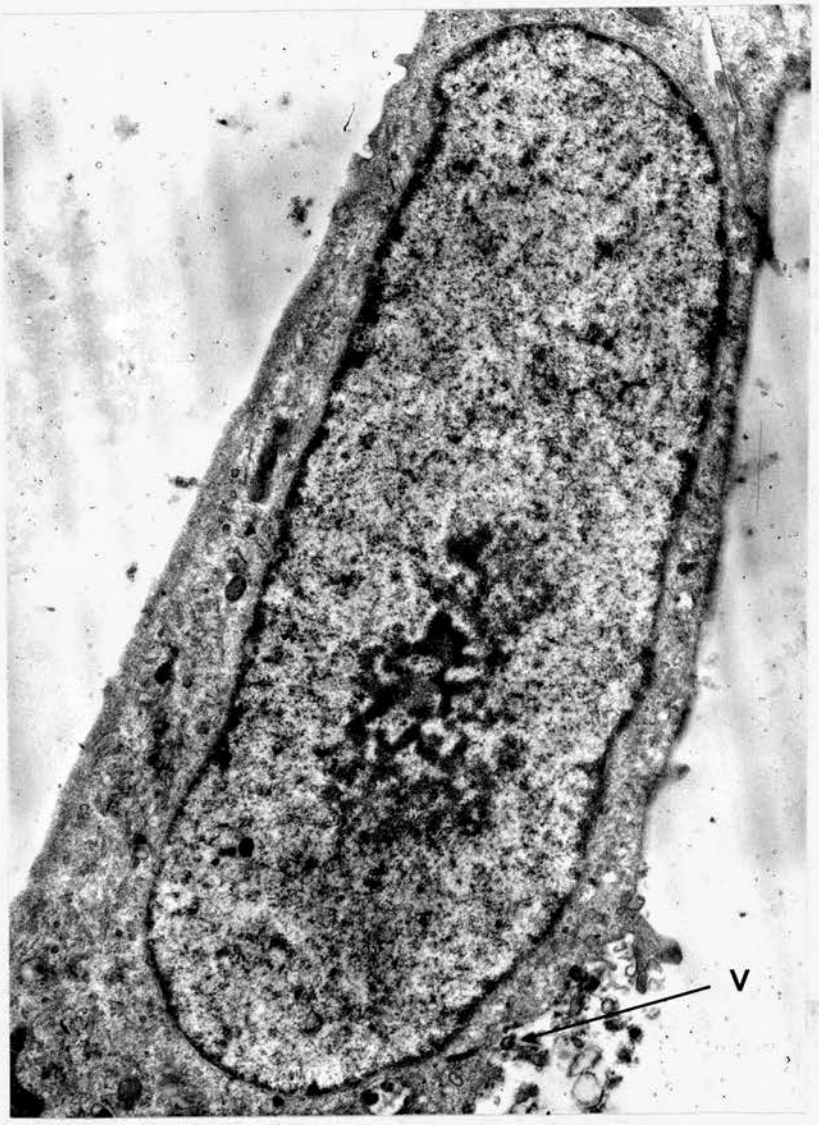


Figure viii (s)

Arginine-free medium: 6 hours post-inoculation.

No cytoplasmic or nuclear changes were observed. In this cell, but not in others, some virus particles (V) were observed still attached to the outside of the cell.

x 12,000

Figure viii (t)

Arginine-free medium: 9 hours post-inoculation.

No virus particles were seen in the nuclei of such cells.

x 26,000

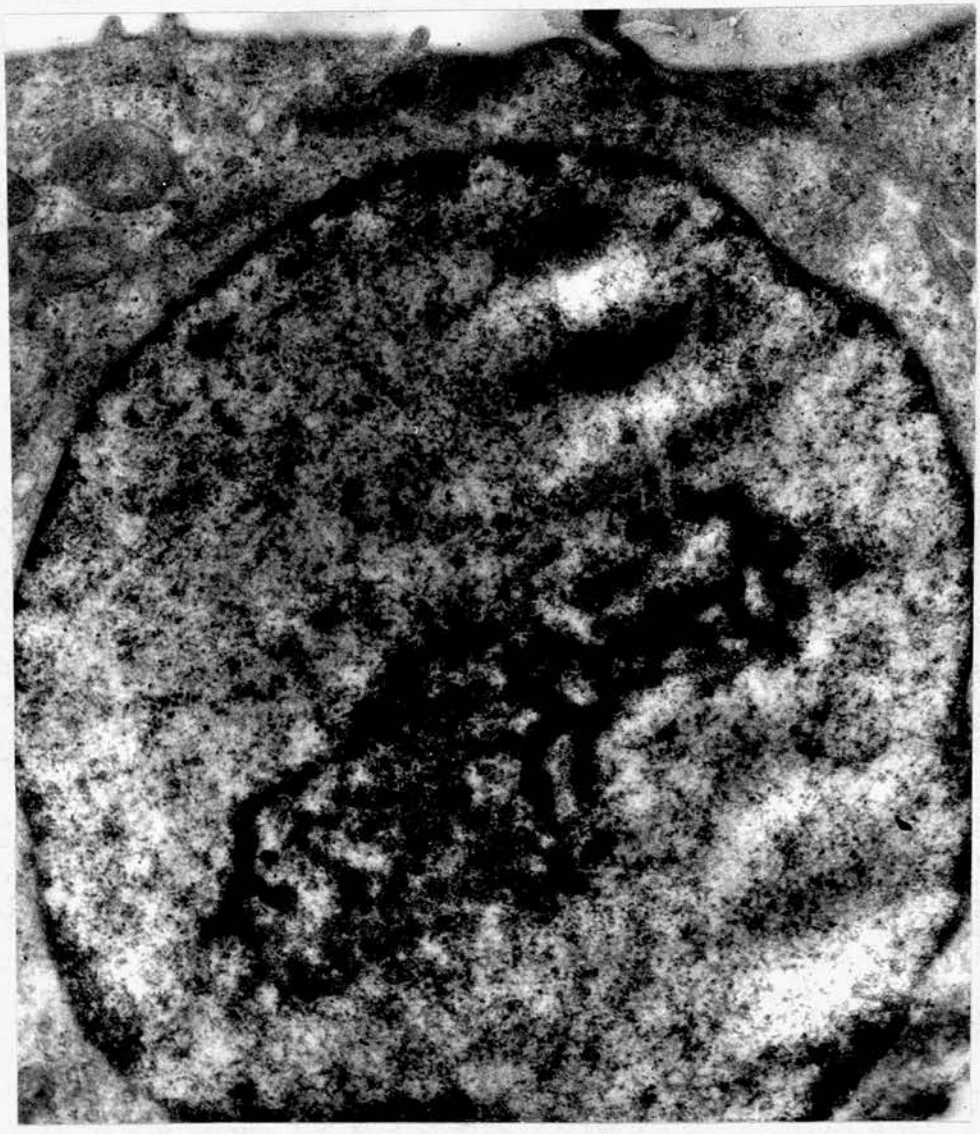


Figure viii (u)

Arginine-free medium: 11 hours post-inoculation.

Nuclei of these cells retained normal appearance. Nucleoli remained intact, there was no margination of the chromatin and no virus particles or virus inclusions were seen in these cells. Compare this with fig. viii (j).

x 26,000

Figure viii (v)

Arginine-free medium: 23 hours post-inoculation.

No nuclear changes were observed. No virus particles or virus inclusions were formed.

x 16,000

The findings from the series of electron micrographs confirm the biological findings that virus adsorbs, penetrates and becomes eclipsed in arginine-free medium. They confirm the results obtained from Giemsa and acridine orange staining, namely, that characteristic nuclear changes occur in herpesvirus-infected cells growing in complete medium, with disappearance of nucleoli and banding of chromatin, but that no such changes occur in infected cells growing in an arginine-free medium.

It was further shown that in normal growth media infected cells start to produce particulate virus in the nucleus at 9 hours post-infection. Virus particles leak from the nucleus without disrupting it, and collect an outer membrane as they pass from the nucleus into the cytoplasm. Virus particles were then seen in various positions in the cytoplasm. The syncytia, characteristic of this virus, begin to form about 11 hours post-infection. No such cellular changes in infected cells growing in arginine-free medium were observed. No virus particles, complete or incomplete, nor any particulate virus component could be observed in these cells.

vii. Estimation of the Time in the Growth Cycle at which Replication was Arrested.

The design of this experiment is most easily seen by considering the results which are illustrated in Figure ix. Tubes of RK_{13} cells were prepared, washed and inoculated in the usual way. The multiplicity of infection was greater than 1 pl. f. u. per cell. One group of cultures received Eag medium; the remaining and larger group of cultures received Arg^- medium. Cultures were incubated at 37°C with rolling. Samples were withdrawn in fours from each group during the first 24 hours and assayed for virus yield, i.e. one-step growth curves in Eag and in Arg^- media were carried out. After the first 24 hours the Arg^- medium was drained from the remaining cultures and Eag medium added to one half of the group, while fresh Arg^- medium was added to the second half. Cultures were reincubated and samples withdrawn at intervals over a second 24 hours, and assayed as before to give results for a second pair of growth curves.

The results of such an experiment are shown in Figure ix.

The object in carrying out such an experiment was to determine if cells containing suppressed virus, when stimulated to resume virus replication, produced new infective virus after a diminished eclipse phase. The decrease in duration of the eclipse phase might then be

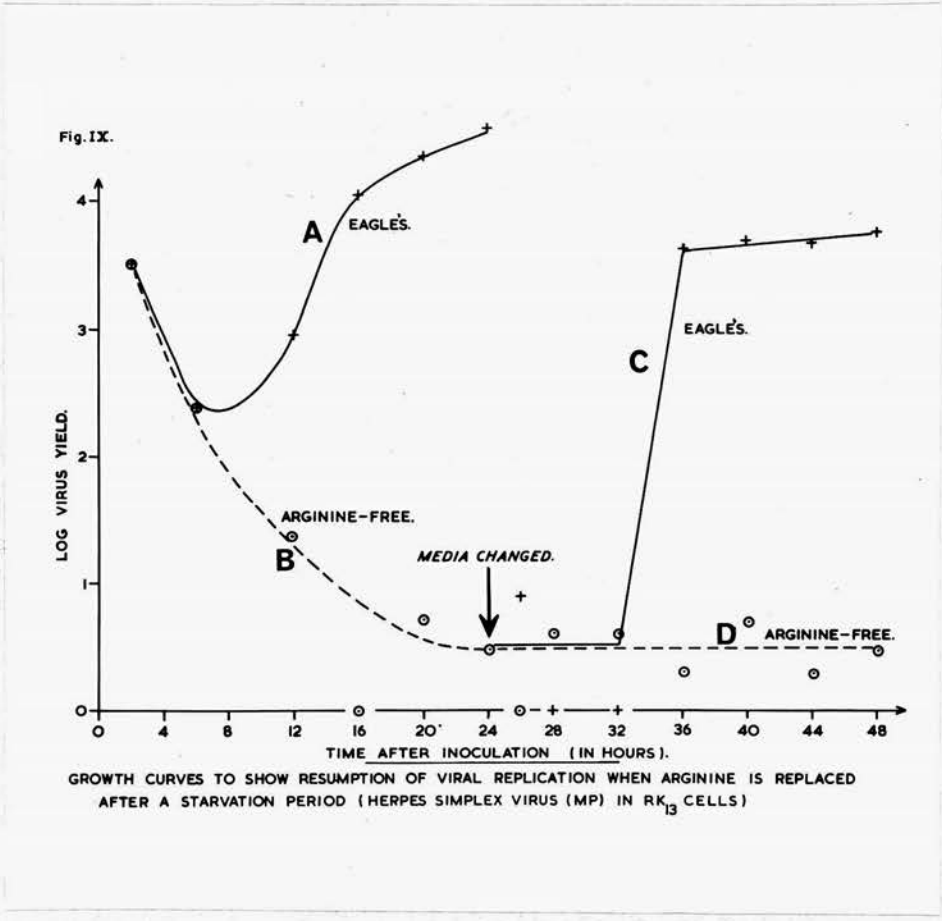


Figure ix. One-step growth curves to show resumption of viral replication upon readdition of arginine to the growth medium.

a reflection of distance along the viral replication pathway already travelled before the deficiency induced stoppage. That the eclipse phase may be shortened was initially suggested by results of the type shown in Table 10, when plaque size of previously suppressed virus reached 'standard' 24 hours quicker than under normal conditions.

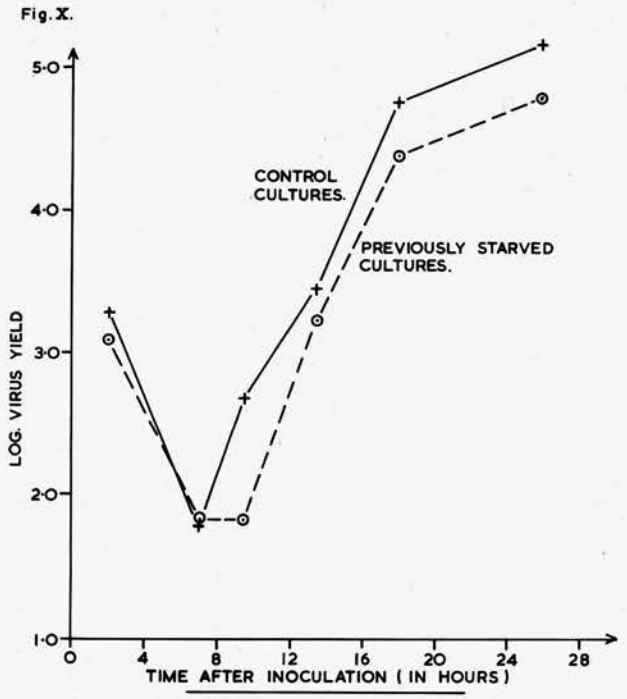
From the normal Eag medium growth curve shown in Figure ix and from other experiments, it was seen that new infective virus begins to be produced 8 hours post-infection. The second growth curve in Eag medium, which represents that of a more synchronous culture, indicates that new infective virus is produced probably between 9 and 11 hours after addition of complete medium. A repeat experiment in which samples were taken at more frequent intervals showed resumption of virus growth after a lag of 9 hours. These results suggested no curtailment of the eclipse phase. Normal and previously suppressed virus had eclipse periods of about 8 and 9 hours duration respectively before new virus was produced.

It was possible, however, that there was a lag before resumption of cellular or viral metabolism upon replacement of complete medium, due to the previous arginine starvation of the cells. This may have been due to the time required for arginine to be absorbed by the cell or else for the synthesis of cellular enzymes

which had become depleted under starvation conditions. This hypothesis was examined by the following experiment.

Two sets of tube cultures of RK_{13} cells were prepared in the usual way. The cultures were drained and washed and then Eag medium was added to one set and Arg^- medium to the other. These were incubated in the usual way for 24 hours, thereafter the cultures were drained, washed and inoculated as usual. All cultures were incubated in Eag medium and samples were withdrawn at intervals and assayed for virus yield. Any difference between the resulting growth curves must have been due to the different preinoculation history of the cultures and not due to different treatment during the period of infection. The results obtained are shown in Figure x.

Results in Figure x show that the final virus yield is lower in cells previously starved of arginine for 24 hours than in normal cells. This confirms the findings illustrated by Figure ix. There is a difference in the two curves shown in Figure x and this is difficult to interpret. It appears that virus is produced after a longer eclipse period in previously starved cells, as compared with normal cells. Therefore, part of the lag period shown in Figure ix is probably due to this cellular effect. An assessment of the significance of these differences will be made later.



THE EFFECT OF INCUBATING CULTURES FOR 24 HOURS IN ARGININE-FREE MEDIUM BEFORE INOCULATION WITH VIRUS.

ONE-STEP GROWTH CURVES OF HERPES SIMPLEX VIRUS (MP) IN RK_{13} CELLS PREVIOUSLY INCUBATED IN EAGLE'S OR IN ARGININE-FREE MEDIUM.

Figure x. One-step growth curves to show the effect on virus growth of prior arginine starvation.

The last group of experiments described suggested that breakdown in virus replication due to an arginine deficiency occurred early in the replication pathway. It was not possible to locate the breakdown point more exactly in this work since this would have required the use of techniques, in particular radioactive tracing, that were outwith the scope of this thesis.

C. The Effect of Some Amino Acid Deficiencies on Other Virus-infected Cell Systems

a) Vaccinia virus in RK_{13} cells

i. Arginine Deficiency.

The growth of vaccinia virus in RK_{13} cells deficient in arginine was investigated and the effect of calf serum supplementation of arginine deficient media also examined.

Monolayer cultures of RK_{13} cells were prepared in petri dishes, washed and inoculated with vaccinia virus according to standard procedures. Overlays with and without Methocel were added, cultures were incubated and then examined for plaque formation or assayed for virus yield as appropriate. Results of two experiments are shown in Table 15. Concentrations of 10 and 20 per cent. calf serum were tested in one experiment and 0-5 per cent. in another. Specimen cultures from the second group were photographed to demonstrate the effect of these media on plaque size. These are shown in Figure xi (a-l).

These experiments showed that in complete medium the concentration of calf serum required for optimal plaque formation and virus production was less critical for vaccinia than for herpesvirus. However, in the complete absence of calf serum, the yield of vaccinia was much reduced. It is further shown in Table 15 that increasing concentrations of calf serum substituted increasingly for arginine in the growth medium and that 20 per cent. calf

Table 15. Growth of vaccinia virus in RK_{13} cells in arginine deficient medium supplemented with various concentrations of calf serum.

Concentration of calf serum (per cent.)	Number of plaques per petri dish in: Eag (approx.)	Arg⁻ (approx.)	Number of pl.f.u. per petri dish in: Eag	Arg⁻
20	620	530	9.4×10^6	11.1×10^6
10	660	370	18.6×10^6	1.0×10^6
5	700	a few	1.4×10^6	1.8×10^4
2	700	a few	1.3×10^6	4.1×10^3
1	600	trace) *	5.8×10^6	1.8×10^3
0	500	trace)	3.4×10^5	1.1×10^3

* These irregularities in the cell sheet could not be identified with certainty as plaques

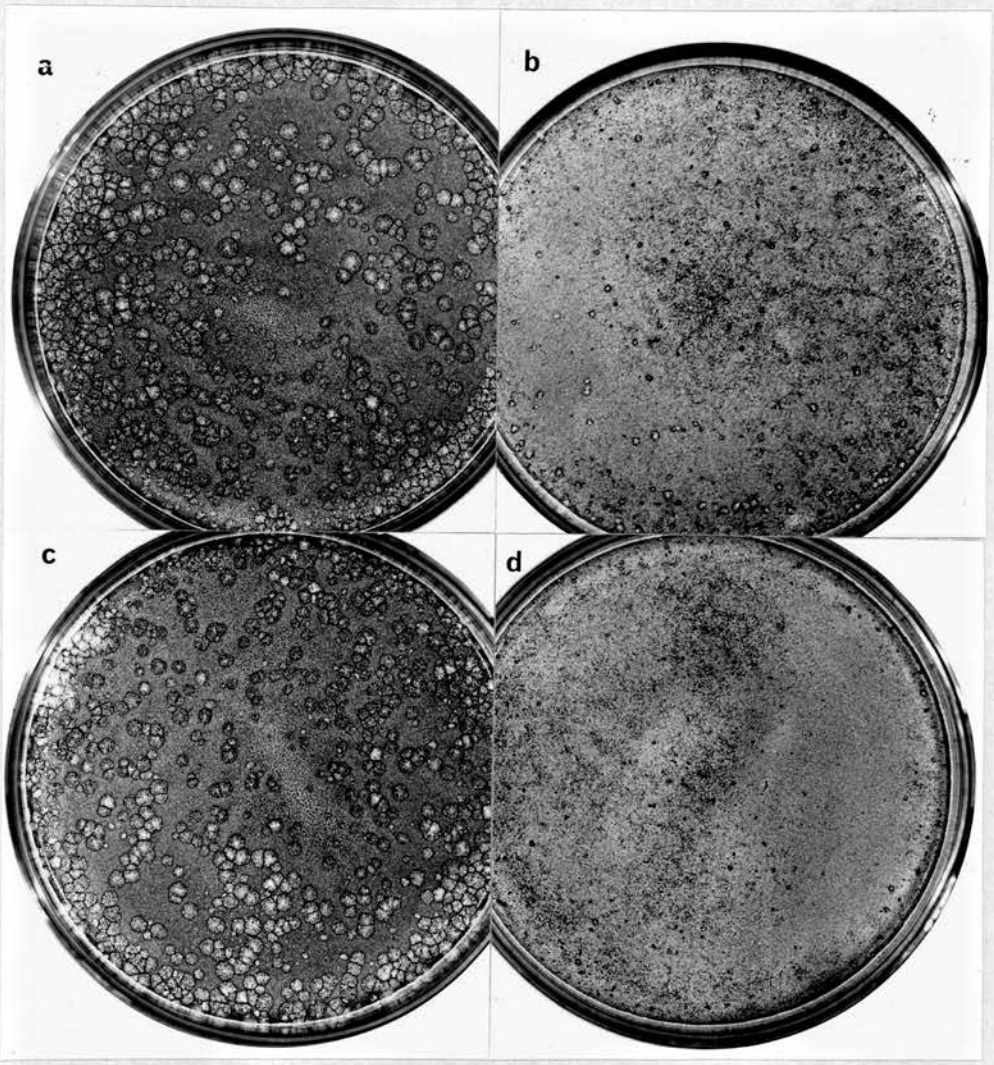
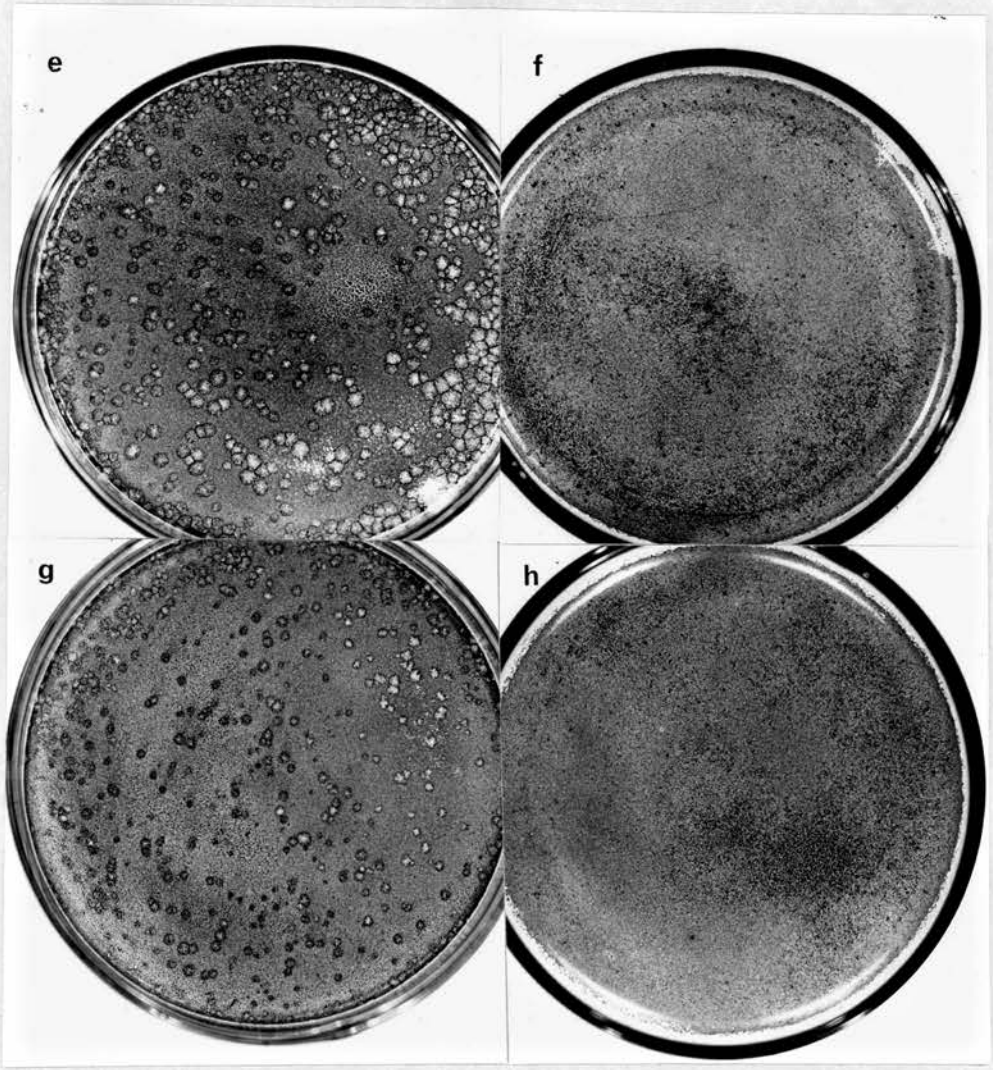


Figure xi (a - h)

The effect of varying concentrations of calf serum in Eagle's medium and in arginine-free medium on plaque formation by vaccinia virus in RK_{13} cells.



a and b: 5 per cent. calf serum Eag and Arg$^-$ respectively.
c and d: 2 " " " " " " "
e and f: 1 " " " " " " "
g and h: no " " " " " "

Petri dishes stained with 0.1 per cent. methyl violet.

Actual size.

serum in an arginine-free medium supported optimal virus yield. Even in the complete absence of arginine there was a small amount of replication of vaccinia virus; this was in contrast to the findings with herpesvirus.

Since the growth of vaccinia was never totally inhibited by the omission of arginine, experiments on the effect of replacing the missing nutrient were of less importance. However, the following experiments were carried out to determine whether replacment of arginine after a period of starvation stimulated the reduced virus reproduction. Preparation of cultures, washing and inoculation were as normal. The effect of replacing complete medium on vaccinia infected cells after a period of arginine deficiency is shown in Table 16.

Table 16. Resumption of growth of vaccinia virus in RK_{13} cells when complete medium was replaced after a period of arginine deficiency.
Media contained 2 per cent. calf serum and 0.75 per cent. Methocel.

Overlay	*Number of plaques per petri dish	Size	Number of pl.f.u. per petri dish
(a) Eag for 48 hours	500	N	10^6
(b) Arg^- for 48 hours	200	$\ll$ N	10^3
(c) As for (b) and then Eag for 48 hours	500	> N	6×10^7

N = normal size.

* Counts were close approximations.

Table 16 indicated that after a period of reduced viral synthesis caused by deficiency of arginine, synthesis increased after the missing amino acid was replaced. The number of plaques formed upon replacement of arginine was approximately equal to the number formed originally on the Eagle's medium control culture. There was a strong probability, nonetheless, that during replacement of the medium there was a spread of extracellular virus leading to initiation of secondary plaques. A second experiment was designed to reduce this source of error.

Cultures were prepared, washed and inoculated. Eag or Arg^- medium containing one per cent. calf serum and Methocel was then added. The procedure which followed is outlined in Table 17 with one added precaution. Cultures to be reincubated after a change of medium were washed with Hanks BSS containing anti-vaccinial antiserum. It was found in other experiments that pre-washing with a standard dilution of this antiserum preparation reduced subsequent initiation of plaque formation to about 20 per cent. It has also been shown (e.g. Hume et al., 1965) that after initiation of infection, development of vaccinia plaques in RK_{13} cell cultures continues in the presence of homologous antiserum. It was hoped, therefore, by washing cultures with antiserum to reduce secondary plaque formation without neutralising any pre-existing foci of infection.

Results are shown in Table 17.

Table 17. Plaque formation by vaccinia virus in RK_{13} cells when complete medium was replaced after a period of arginine deficiency. Media contained 1 per cent. calf serum and 0.75 per cent. Methocel.

Overlay	Number of plaques per petri dish
(a) Eag for 48 hours	67
(b) Arg^- for 48 hours	*
(c) As (a) then Eag for 48 hours	94
(d) As (b) then Eag for 48 hours	83

* Trace lesions only.

Results shown in Table 17 suggest that secondary plaque formation was not completely eliminated and that approximately 27 plaques, i.e. (c-a), were formed per culture as a result of the washing procedure. The number of plaques on culture (d) due to original infection would then be 56 per petri dish, i.e. (d-27), and this is equal, within the limits of experimental error, to the initial count obtained from the control Eagle's culture. Therefore, under the conditions of this experiment each original focus of infection resumed development after a nutrirional deficiency when the missing nutrient was replaced.

ii. Lysine Deficiency.

Since it had been found that the omission of lysine from a growth medium had no adverse effect on the growth of HSV(MP) in RK_{13} cells, it was of interest to assess the effect of this omission on vaccinia virus replication in these cells.

Cultures of cells were prepared, washed and inoculated with vaccinia virus according to standard procedure. The effect of a lysine deficiency on plaque formation and virus production was then assessed.

Results obtained are shown in Table 18.

Table 18. Growth of vaccinia virus in RK_{13} cells in a lysine-free medium.

Growth medium	Number of plaques per petri dish	Number of pl.f.u. per petri dish
Eag	188	4.3×10^6
Lys^-	37*	6.0×10^3

* These were not normal plaques, but only heaps of cells.

Results shown in Table 18 indicate that vaccinia virus replication was greatly reduced although not completely stopped, when lysine was omitted from the growth medium. These results are in contrast to the findings with herpesvirus.

b) Coxsackie B_3 virus in Hela cells

Preliminary studies showed that coxsackie B_3 virus did not grow in RK_{13} cells. Nutritional studies were therefore carried out in a HeLa cell line since this was found to be susceptible to the virus. These findings cannot be compared directly with the herpesvirus and vaccinia virus results obtained from studies in RK_{13} cells.

Preparation of monolayers, washing and inoculation were as for experiments with RK_{13} cells. Overlays for determination of plaque formation contained 1.2 per cent. noble agar. Several experiments were carried out to investigate plaque formation by this virus in a range of media. Representative results are shown in Table 19.

In experiments with coxsackie B_3 virus, there was a large variation in plaque size within a single culture. This variation did not depend on the concentration of calf serum even in complete medium. The number of plaques formed in a culture varied with calf serum concentrations and reduced counts were obtained in the total absence of calf serum. The reduction in count was less marked in complete than in deficient media. However, it was clear from these experiments that HeLa cells could support replication of coxsackie B_3 virus, albeit to a limited extent, in media which contained no arginine nor any lysine.

Table 19. Growth of coxsackie B_3 virus in HeLa cells in complete and in deficient media.

Concentration of calf serum in the medium	Number of plaques per petri dish in:		
	Eag	Arg^-	Lys^-
(per cent.)			
10	145	79	148
5	133	85	109
1	121	36	28
0	53	15	8

3. VIRAL INTERFERENCE

When experiments were being carried out to investigate the state in which latent herpesvirus existed inside arginine deficient cells, the idea occurred to examine the ability of such suppressed virus to cause viral interference. It was necessary to find a virus which, firstly, would grow under conditions of reduced arginine concentration and, secondly, which failed to grow in cells already occupied by herpesvirus. The first condition was fulfilled by coxsackie B_3 virus; however this virus did not grow in RK_{13} cells. It was possible that coxsackie B_3 virus in HeLa cells may have served as a model, but it was considered preferable to continue this work in RK_{13} cells since the HSV(MP)-RK_{13} system had by now been studied in some detail and could be controlled fairly closely. The first condition for a challenge virus appeared to be satisfied by vaccinia virus in RK_{13} cells maintained in Arg^- medium supplemented with 20 per cent. calf serum. The second condition, viz. that HSV(MP) could interfere with vaccinia, was investigated.

Interference experiments were carried out in tube cultures of RK_{13} cells using live HSV(MP) and vaccinia virus both live and heat killed. It had been reported by Galasso and Sharp (1964) that heat killed vaccinia virus interfered with homologous virus in Earle's L

cells. This was investigated since it offered a means of detecting interference of herpesvirus replication by vaccinia virus.

Tube cultures of RK_{13} cells were prepared and inoculated. Multiplicities of infection with the interfering virus, the first virus to enter the cell, and the challenge virus, the second virus to enter the cell, were both calculated at 2 pl. f. u. per cell. The outline of an experiment to determine interference between:

i. heat killed and live vaccinia virus;

ii. heat killed vaccinia and live HSV(MP);

iii. HSV(MP) and live vaccinia;

is indicated in Table 20. Interference was measured by a depression in the yield of the challenge virus.

The results shown in Table 20 indicate that in RK_{13} cells:

i. heat killed vaccinia does not interfere with subsequent challenge by live vaccinia; there may even have been a slight increase in virus yield in cultures pre-treated with heat killed virus;

ii. heat killed vaccinia virus does not interfere with the replication of HSV(MP);

iii. but HSV(MP) does interfere with vaccinia virus, the yield of vaccinia virus being reduced more than tenfold by previous inoculation with herpesvirus.

The fact that HSV(MP) virus interfered with the

Table 20. Interference between HSV(MP) and vaccinia virus.

Interfering inoculum	Challenge inoculum	Virus yield per tube in pl.f.u.
(i)		
Vac. K.	skim milk	540 (Vac.)
skim milk	Vac. L.	8,600,000 (Vac.)
Vac. K.	Vac. L.	10,000,000 (Vac.)
(ii)		
Vac. K.	skim milk	540 (Vac.)
skim milk	HSV(MP)	1,000 (HSV)
Vac. K.	HSV(MP)	4,000 (HSV)
(iii)		
HSV(MP)	skim milk	1,000 (HSV)
skim milk	Vac. L.	8,600,000 (Vac.)
HSV(MP)	Vac. L.	680,000 (Vac.)

Vac. K. = heat killed vaccinia (56°C for 45 min)

Vac. L. = live vaccinia

growth of a challenge inoculum of vaccinia virus, and an early observation that RK_{13} cells maintained in 20 per cent. calf serum Arg^- medium, could not support replication of HSV(MP) but supported the growth of vaccinia to a normal level, suggested that this system might be suitable to investigate interference by latent HSV(MP). However, it was later found that although cells maintained in Arg^- medium with 20 per cent. calf serum did not support herpesvirus growth when they were infected at very low multiplicities, when all the cells in a population were infected there was production of some new infective HSV(MP). Thus in the suggested interference system it would not be possible to distinguish between interference due to latent virus and that due to normally growing herpesvirus. This system, therefore, was developed no further.

The interference of HSV(MP) by live and ultra-violet inactivated vaccinia was investigated. The ultra-violet inactivation process was described on page 121 and reduced the infectivity of the virus preparation 100-fold. Tests were carried out on RK_{13} monolayers in petri dishes. Cultures were inoculated with vaccinia virus, live or ultra-violet inactivated, and adsorption continued for 2 hours at 37°C. The residual inoculum fluid was then drained off and the challenge inoculum of HSV(MP) added. After a further 2 hours adsorption period the second inoculum fluid was removed, overlay was added and

cultures incubated and examined for plaque formation. Controls were included in which the initial vaccinia inoculum was replaced by sterile skim milk to give the normal yield of herpesvirus plaques and also in which the herpesvirus inoculum was replaced by skim milk to give the normal yield of vaccinia virus plaques. Plaques formed by vaccinia virus and HSV(MP) are quite distinctive and can be differentiated easily. Results obtained are shown in Table 21.

In the first two columns of Table 21 results are shown of cultures inoculated with vaccinia virus only. As shown some plates received inocula containing live vaccinia while others received ultra-violet inactivated inocula containing some residual live virus. The second two columns show the results of the interference experiment. Primary inoculation with live vaccinia or with ultra-violet inactivated vaccinia, exactly as in the controls, was followed by challenge inoculation with herpesvirus. The final column shows the number of plaques formed on the 'herpesvirus only' controls.

It can be seen from these results that live vaccinia interferes with herpesvirus growth even when the inoculum of vaccinia is low, e.g. 7 pl. f. u. per culture. The interference, as measured by percentage suppression of herpesvirus plaques, was not directly proportional to the size of the vaccinia inoculum. Finally, it can be seen that ultra-violet inactivated vaccinia did not interfere with the growth of herpesvirus.

Table 21. Interference between HSV(MP) and vaccinia viruses.

Number of plaques per petri dish formed in:				
Vaccinia cultures		Mixed cultures		Herpesvirus cultures
Normal vaccinia	u/v inactivated vaccinia*	Vaccinia	HSV(MP)	HSV(MP)
420	-	SC	200	380
56	-	45	231	380
10	-	7	290	380
31	4000	23	266	380
4	400	1	400	380
1	40	1	400	380

* These counts represent the calculated number of ultra-violet inactivated virus particles per inoculum

- = No u/v particles in the inoculum

SC = Semi-confluent.

Counts of 400 or more plaques per petri dish are approximations.

4. HEPARIN STUDIES

It has been reported by several workers (Vaheri, 1964; Nahmias and Kibrick, 1964) that heparin is active against several strains of herpesvirus and that it exerts its effect either by preventing the initial attachment of the virus particle to the cell or else prevents some other very early part of the virus replication cycle. The object of these studies was to determine whether the HSV(MP) strain of herpesvirus was susceptible to the action of heparin, and if so to determine the reversibility of this reaction, and then to investigate this as a possible mechanism of the extracellular type of latency suggested in the introduction to Part II of this thesis.

A. The Antivial Action of Heparin

a) The extracellular effect of heparin

The extracellular effect of heparin on HSV(MP) and on vaccinia virus was investigated. A range of heparin dilutions, prepared in Hanks BSS, was mixed at room temperature with virus suspensions in pyrex Wasserman tubes. Aliquots of each mixture were withdrawn and titrated on RK_{13} monolayers without further dilution. The results of such an experiment are shown in Figure xii.

It is shown in Figure xii that HSV(MP) was

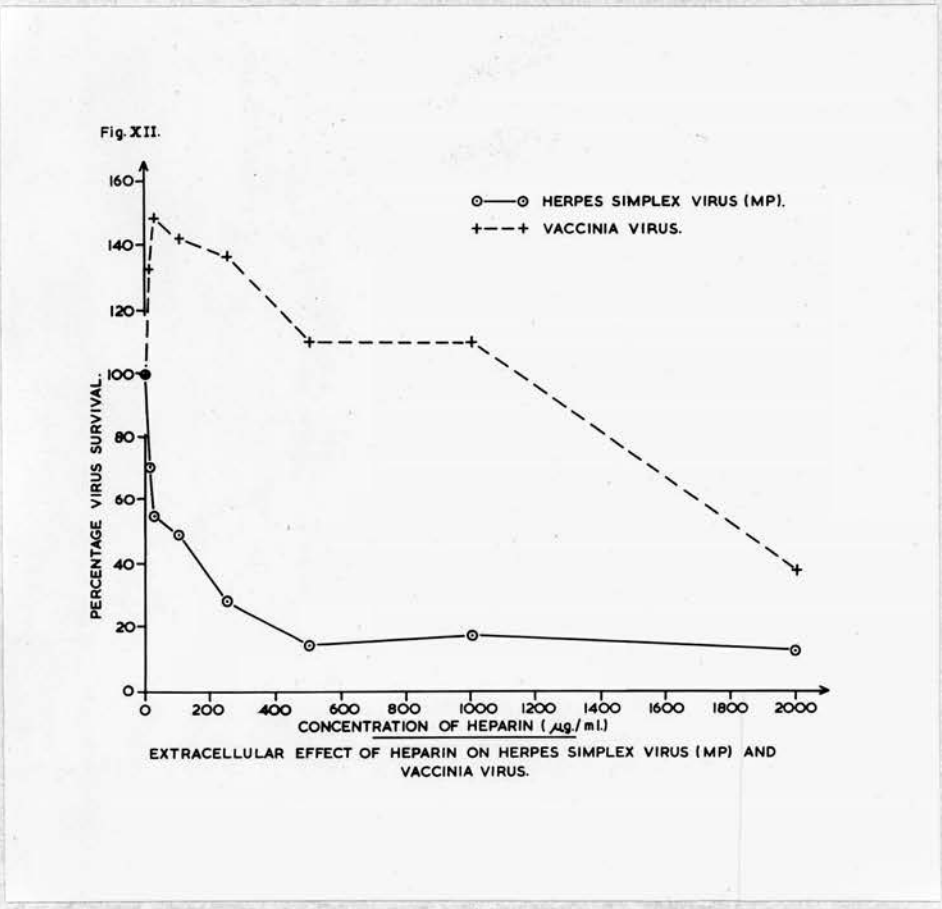


Figure xii. To show the effect of mixing heparin with Herpes simplex virus and vaccinia virus prior to inoculation of virus on to RK_{13} cells.

inactivated increasingly with increasing concentrations of heparin up to 500 µg per ml; above this concentration no further inactivation was achieved. It has been shown in many experiments that there is always a resistant fraction in a herpesvirus population, of the order of 10-15 per cent. Concentrations of heparin up to 1000 µg per ml did not inactivate vaccinia virus. With vaccinia preparations a higher titre was obtained in the heparin treated samples than in the untreated, suggesting that heparin exerted a protective effect on the virus. There was some inactivation of vaccinia when heparin at a concentration of 2000 µg per ml was used. This concentration appeared to have an adverse effect on the cells.

b) The intracellular effect of heparin

Intracellular action of heparin on HSV(MP) and on vaccinia virus was investigated. This effect of heparin was studied by allowing the virus to interact with the cells for a given period and then adding heparin to the complex.

Petri dish monolayers of RK_{13} cells were prepared and inoculated with HSV(MP) diluted in Hanks BSS. Adsorption was continued for 3 hours at 37°C, after which residual inoculation fluid was removed and the cultures washed twice with Hanks BSS. Growth media containing a range of heparin concentrations were added to the cultures. No Methocel was used. Control cultures which contained no heparin were included; two of these cultures contained Methocel and two did not. Cultures were incubated for 60 hours at 37°C and then examined for heparin-induced cellular damage, and for plaque formation. The results obtained are shown in Figure xiii.

Figure xiii shows percentage of plaque formation plotted against heparin concentration. Two curves were drawn. One was based on percentages calculated from the Methocel-containing control on which there was no secondary plaque formation, and the other on percentages calculated from the Methocel-free control. The significance of these two curves will be discussed later. The third curve shown on Figure xiii was drawn

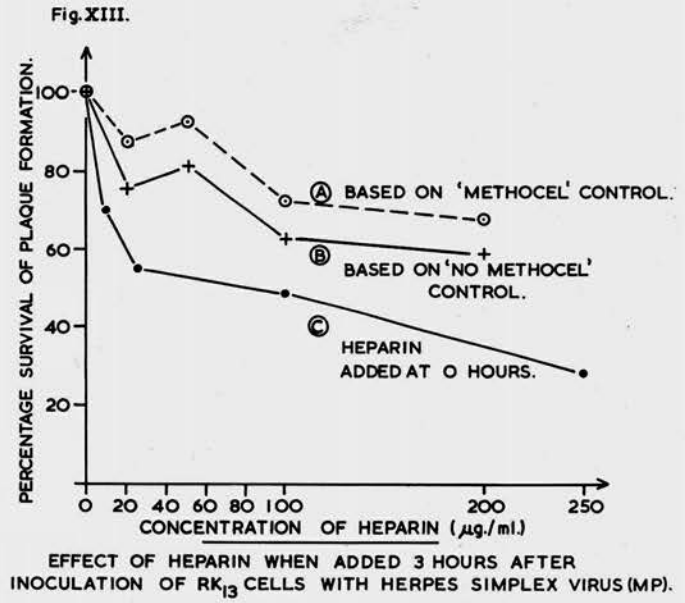


Figure xiii. To assess the effect of heparin on the intracellular replication of Herpes simplex virus.

from data given in Figure xii, and was included to compare the action of heparin on herpesvirus when mixed with free virus and when added after 3 hours of host cell-virus interaction. From these results it was clear that heparin was effective against some very early stage of the virus replication cycle, perhaps even initial attachment, and that the effect of heparin was considerably diminished if added 3 hours post-inoculation. However, it was observed that heparin concentrations of 50 µg per ml and greater, when added 3 hours after infection with virus, caused a reduction in plaque size. There was a suggestion of an anticellular effect by heparin at 200 µg per ml. At this concentration the monolayers appeared sparser after 60 hours incubation than did the controls. The effect was not marked.

The antiviral action of heparin on vaccinia virus infected cells was investigated in a similar manner. Heparin containing media were added to cultures after 3 hours normal adsorption at 37°C. Controls without heparin were included. All media contained Methocel.

Results obtained are shown in Figure xiv.

In Figure xiv the percentage survival of the ability to form plaques is plotted against concentration of heparin in the medium when added 3 hours after inoculation. Results on the extracellular effect of heparin obtained from Figure xii are included for comparison. It was seen from Figure xiv that heparin suppressed

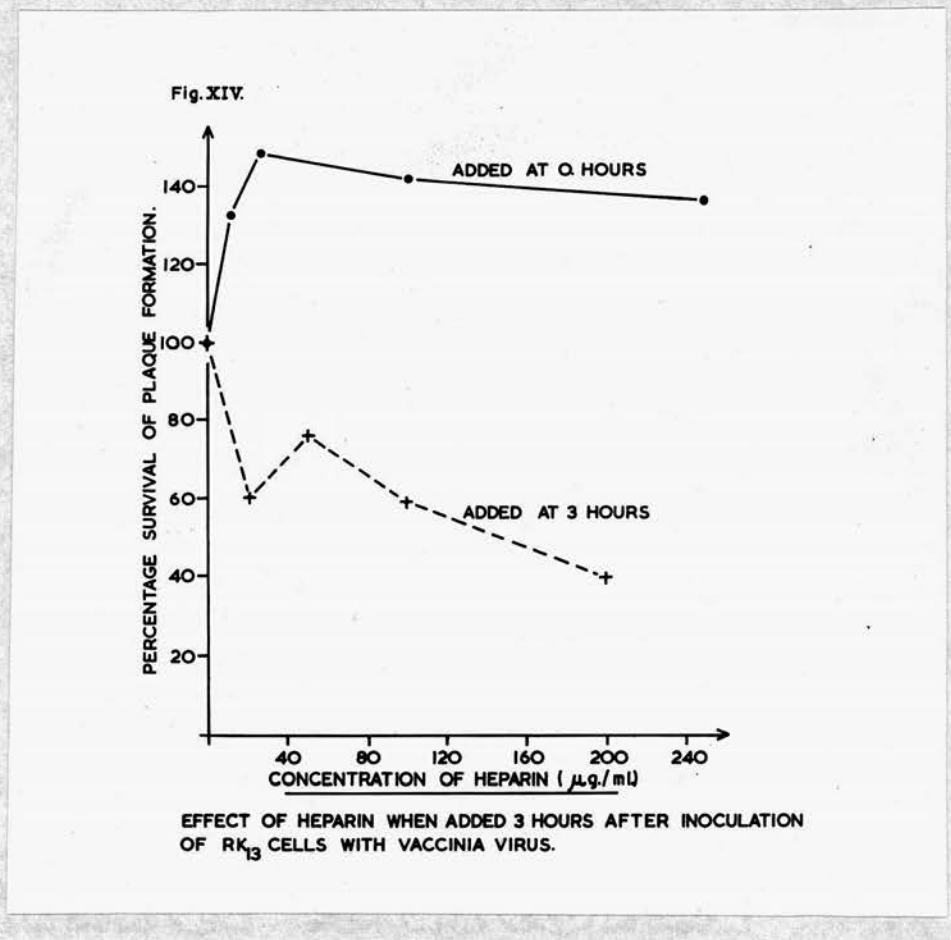


Figure xiv. To assess the effect of heparin on the intracellular replication of vaccinia virus.

plaque formation by vaccinia virus when added after the virus had adsorbed and penetrated. Plaques formed in the presence of 200 µg per ml heparin were minute, being little more than heaps of cells. Plaque size increased as the concentration of heparin decreased and was about normal in the presence of 20 µg per ml heparin.

B. The Antiviral Action of Protamine Sulphate

In order to develop the extracellular inactivation of herpesvirus by heparin as a model of a latent infection, it was necessary to investigate methods of reversing the virus inactivating effect of heparin so that virus could be freed to initiate infection after a period of suppression. It has been reported by Nahmias and Kibrick (1964) and others that protamine sulphate combines with heparin and so inactivates the blood coagulating ability and anti-herpes activity. The possibility was investigated, therefore, that heparin might have a stronger affinity for protamine sulphate than it did for herpesvirus and that protamine might then be used to break heparin-virus complexes and so free the virus to initiate infection of a cell.

Firstly the antiviral effect of protamine sulphate on HSV(MP) was investigated. The effect on vaccinia was also investigated to parallel the interesting results obtained in the heparin studies.

Experimental procedure was similar to that used in experiments on the extracellular effect of heparin. Virus suspensions were mixed with dilutions of protamine sulphate in Hanks BSS for 15 minutes at room temperature and aliquots of each specimen titrated by the plaque technique for residual virus infectivity. Results are shown in Figure xv in which the percentage survival of

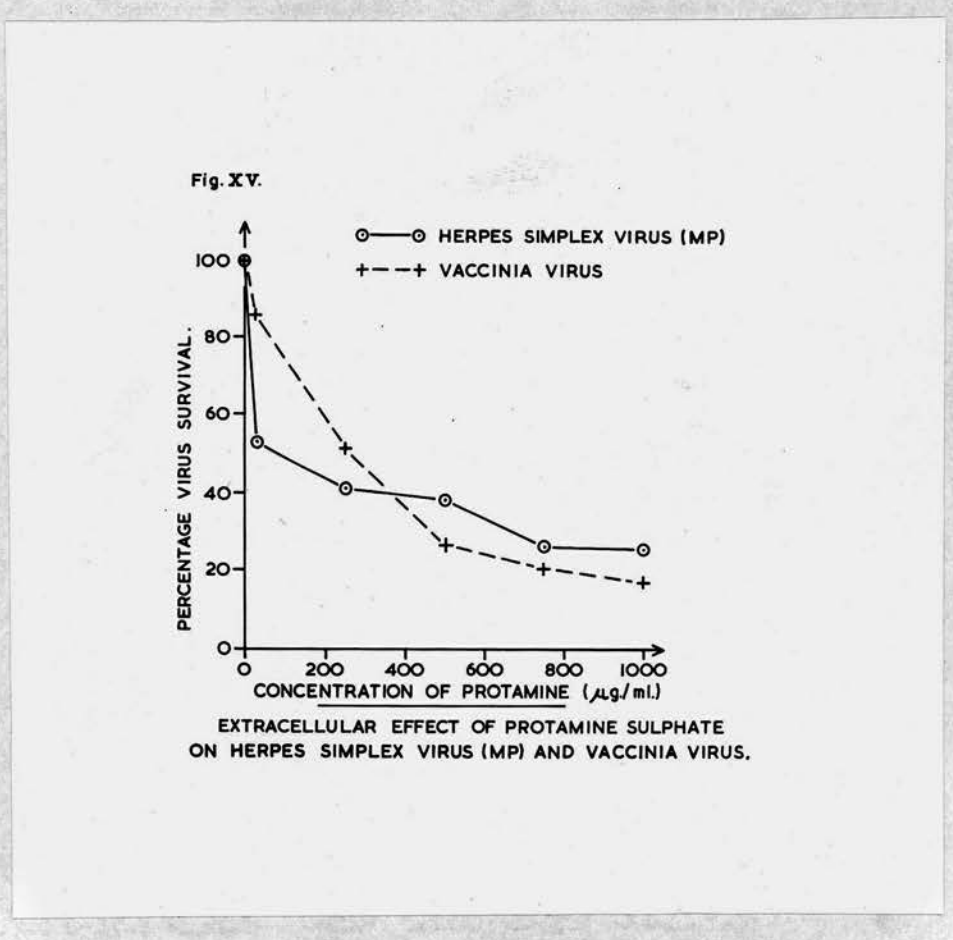


Figure xv. To show the effect of mixing protamine sulphate with Herpes simplex virus and vaccinia virus prior to inoculation of virus on to RK_{13} cells.

plaque forming ability is plotted against the concentration of protamine sulphate in the suspending fluid.

Figure xv indicates that protamine sulphate inactivated both HSV(MP) and vaccinia when mixed with these viruses before inoculation on to cell monolayers. Inactivation increased with protamine concentration and the degree of inactivation was approximately equal for both viruses. In these experiments there was no effect on the plaque character of herpesvirus, but vaccinia plaques were small and indistinct when the virus had been suspended in concentrations of 750 µg per ml or greater. Protamine carried over in the inoculum exerted an anticellular effect when the mixture concentration was 750 µg per ml or greater; such monolayers after 48 hours incubation appeared sparser than normal.

A comparison of the dose-response curves of heparin and protamine with herpesvirus showed that the two compounds inactivated this virus at approximately equal rates. It is unlikely, therefore, that infective herpesvirus would be liberated from a virus-heparin complex by means of protamine. However, because of the difference in effect of these two compounds on herpesvirus and on vaccinia it is possible that the mode of action of the two inhibitors is different, and that each might neutralise the antiviral action of the other preferentially. It was considered worth trying a few

pilot experiments to investigate the effect of protamine on a heparin-herpesvirus complex.

C. Recovery of Infectivity of HSV(MP) from a Heparin-Virus Complex

a) By protamine sulphate

A series of experiments were carried out in which heparin was mixed with a herpesvirus preparation for a given time, and then protamine sulphate was added to the mixture. Finally aliquots were assayed for resultant viral infectivity. Experimental procedure and necessary controls are indicated with the results shown in Table 22. Tests were carried out at room temperature in pyrex Wasserman tubes. One test will be given as an example.

Heparin was diluted in Hanks BSS and 1 ml was added to 1 ml of a dilution of herpesvirus in Hanks BSS. This was gently mixed for 5 minutes by manual shaking. Then 1 ml of the mixture was added to 1 ml of a dilution of protamine and this shaken for 10 minutes. Aliquots were then withdrawn and titrated. The final concentrations of heparin and protamine are shown in Table 22.

Results shown in Table 22 indicate that herpesvirus can be freed from the inactivating effect of heparin by later addition of protamine sulphate. However, it appears that the relative concentrations of the two compounds are very important if the antiviral action of both these agents is to be neutralised. The results of experiment A shown in Table 22 support the suggestion

that the affinity of heparin for protamine is greater than for virus.

Table 22. Recovery of infectivity of HSV(MP) from a heparin complex by means of protamine sulphate.

Treatment of sample	Percentage survival of virus infectivity A	B
Hanks + virus (5 min) then Hanks (10 min)	100	100
Heparin + virus (5 min) then Hanks (10 min)	10	3
Hanks + virus (5 min) then protamine (10 min)	19	35
Heparin + virus (5 min) then protamine (10 min)	87	20

Concentrations in the final mixture were:

A: heparin 100 µg per ml, protamine sulphate 200 µg per ml

B: heparin 100 µg per ml, protamine sulphate 100 µg per ml

Another experiment of this type was carried out using longer reaction times and lower concentrations of reagents. Experimental details and results are shown in Table 23.

Table 23 again demonstrates the recovery of herpesvirus infectivity from antiviral action of heparin by use of protamine sulphate.

Table 23. Recovery of infectivity of HSV(MP) from a heparin complex by means of protamine sulphate.

Treatment of sample	Percentage survival of virus infectivity
Hanks + virus (15 min) then Hanks (15 min)	100
Heparin + virus (15 min) then Hanks (15 min)	7
Hanks + virus (15 min) then protamine (15 min)	78
Heparin + virus (15 min) then protamine (15 min)	118

Concentrations in the final mixtures were:

Heparin 50 µg per ml, protamine sulphate 50 µg per ml

There is the objection to the experiments described in Tables 22 and 23 that the effect of the protamine is simply to shorten the effective time of action of heparin by neutralising that heparin which has not yet reacted with the virus. Experiments were carried out, therefore, to investigate the neutralisation of the antiviral action of heparin and protamine by admixture of the two compounds before they came in contact with the virus.

Tests were again carried out at room temperature in pyrex Wasserman tubes. Equal volumes of dilutions of heparin and protamine were mixed by moderate manual agitation before addition to virus preparations. In experiments A, B and C indicated in Table 24 the period

of heparin-protamine mixing was 10 minutes. In experiment D it was 15 minutes.

Table 24. Effect on the antiviral actions of heparin and protamine sulphate by mixing these two agents.

Treatment of sample	Percentage survival of virus infectivity A	B	C	D
Virus + Hanks	100	100	100	100
Virus + heparin	12	14	6	17
Virus + protamine	54	31	55	69
Virus + heparin-protamine mixture	62	33	14	28

Concentrations in the final mixtures were:

A: heparin 50 µg per ml, protamine 50 µg per ml

B, C, D) heparin 200 µg per ml, protamine 200 µg per ml

B, C and D were replicate experiments.

The results shown in Table 24 indicate that admixture of heparin and protamine does not eliminate their antiviral activities and that this is more marked at high concentrations. This result was unexpected since it has been shown, see Tables 22 and 23, that protamine can release virus from the inhibitory effect of heparin.

b) Recovery of virus infectivity from a heparin complex by dilution

It had been reported by Nahmias and Kibrick (1964) that, in their system, the inhibitory effect of heparin could be reduced by simple dilution. This is a possible mechanism which might operate in a latent system. When the heparin concentration in a system decreases, virus may then be freed to initiate infection and conversely when the heparin concentration at the site of infection increases it may be effective in restricting the infection. The reversal of the effect of heparin by dilution was investigated.

An aliquot of herpesvirus suspension was added to each of a series of pyrex Wasserman tubes; an equal volume of heparin solution was added to one set of tubes to give a final concentration of 1000 µg per ml and an equal volume of Hanks BSS added to a second set as controls. The mixtures were incubated with periodic shaking for 30 minutes either at 37°C or 4°C. Samples were then withdrawn and titrated on RK_{13} monolayers either with or without subsequent dilution. The results obtained are shown in Table 25.

Results shown in Table 25 indicate that heparin at a concentration of 1000 µg per ml exerted an inhibitory effect on HSV(MP). Upon ten-fold dilution of the test mixture the inhibitory effect was still observed but in this sample the heparin concentration in the inoculum

Table 25. Recovery of infectivity of HSV(MP) from the inhibitory effect of heparin by dilution.

Concentration of heparin in inoculum	Sample held for 30 min. in: heparin (1000 µg per ml)	Hanks BSS
(a) test carried out at 37°C		
1000 µg per ml	SC	Total destruction of cell sheet
100 "	500 *	SC
20 "	141	169
10 "	17	20
1 "	1	1
(b) test carried out at 4°C		
1000 µg per ml	SC	Total destruction of cell sheet
100 "	500	SC
20 "	156	168
10 "	22	25
1 "	1	1

SC = semi-confluent CPE, probably several thousand foci of infection

* Counts of 500 or greater are only close approximations.

was 100 µg per ml. At higher dilution when the concentration of heparin in the inoculum was 20 µg per ml or less there was no reduction in virus titre thus suggesting that any complex formed by incubation with 1000 µg per ml heparin had been dissociated on dilution.

PART II

A Tissue Culture Model of a Latent Infection with Herpes Simplex Virus

DISCUSSION

1. Selection of Test System

There are two distinct types of plaque formation caused by different strains of Herpes simplex virus. These have been described as macro-plaques (MP) and micro-plaques (mP) by Roizman and Roane (1963). Since the latter type of lesion is difficult to identify with certainty among the irregularities normally occurring in a cell sheet, it was considered preferable to work with a stable stock of MP virus.

A wild strain of Herpes simplex virus, designated strain 244, had been isolated from a throat swab, and found to produce mP lesions regardless of the cultural history as far as it was studied here. It has been observed (J.F. Peutherer, personal communication) that, in general, wild strains of herpesvirus produce micro-plaques and only very rarely does a macro-plaque develop in these populations. The MP type of virus selected for this work is, in one aspect at least, genetically different from the majority of wild strains of herpes-virus. The advantages in using such a virus type were considered sufficient to justify the choice although this reservation should be noted.

The HFEM strain of Herpes simplex virus was examined and found to produce both MP and mP lesions. The proportions of the two varied according to how the virus was grown. When the virus was propagated in eggs the MP type of virus predominated, whereas when the

virus was serially subcultured in tissue culture cells the mP type of virus emerged and became dominant (see Table 1). No attempt was made to clone the HFEM strain and so propagate a macro-plaque stock of virus. Other workers using this virus, e.g. Watson and Wildy (1963), used a cloned strain no further than three passes from the egg thus suggesting that the emergence of mP variants could be avoided by this means. Because of the experimental procedure used in much of this work it would have been necessary to use a virus stock one or at most two passes away from the egg. It was more convenient here to produce the virus stock in tissue culture and so an MP strain of Herpes simplex virus obtained from Dr. B. Roizman was used for all further studies. There was some minor variation in plaque types formed by populations of this virus but all plaques were of the MP type.

Another observation made during the selection of the test system was the failure of coxsackie B_3 virus to grow in RK_{13} cells although it grew in both HeLa and HEp-2 cells. This was probably due to the absence of specific virus receptors on the surfaces of insusceptible cells as reported by Crowell (1963), and is consistent with the findings of Holland (1961) and with results reported in Part I of this thesis.

2. Nutritional Requirements for Cell and Virus Growth

Much work has been done in the past to investigate the essential nutritional requirements for the growth of tissue cells *in vitro* (Morgan *et al.*, 1950; Eagle, 1955a,b,c, 1956a). It has been found that a wide range of amino acids, salts, vitamins and some sugars is necessary for cell growth. If any one of these essential nutrients is omitted from the medium, the cells stop multiplying and eventually die. Depending on the specific growth factor omitted, from 1-14 days are required for the degenerate effects on the cells to become fully evident (Eagle and Habel, 1956). A generation time of between 10 and 24 hours is found for a large number of viruses. It thus becomes possible to determine which of the nutrients, known to be necessary for survival and growth of the cell, are also essential for the propagation of the infecting virus.

In an investigation of specific nutrient requirement, carried out by omitting selectively single components of the growth medium, three factors become inseparably involved. These are, the absolute cellular requirement, the absolute requirement for viral synthesis, and the size of free intracellular pool of the nutrient.

Some measure of the first can be obtained from cell survival studies in the absence of the nutrient and, in the case of amino acids, from an analysis of the amino

acid composition of total cell protein. Analyses of cell protein of several types of human cells were made by Piez and Eagle (1958). They found that the amino acid present in the highest proportion was arginine, and then lysine, with histidine intermediate and cystine in lowest proportion. The results from all cell types tested were comparable. In particular the analyses of the intracellular pools, which will be discussed below, were similar to findings of other workers on other mammalian cells, e.g. Tallan et al. (1954) working on cat liver cells. It appears reasonable to suggest that there may be some conformity among mammalian cells and that perhaps a somewhat similar situation may exist in rabbit kidney cells. This, if it were so, and the comparatively short survival time of RK_{13} cells in arginine-free medium found during these studies, would suggest a high cellular requirement for arginine.

Piez and Eagle (1958) also analysed the free amino acid pools of several types of human cells. They found only trace amounts of arginine and cystine and low concentrations of histidine. When the conjugated amino acids of the intracellular pools were released by acid hydrolysis and analysed, the component present in lowest concentration was again arginine. Comparable findings have been obtained from analyses of other mammalian cells; in particular it was common that the concentration of arginine in the intracellular pool was very low.

In experiments using deficient media the composition of the intracellular pool will depend on the duration of the period of starvation of the nutrient under study. Piez and Eagle (1958) found that after 24 hours in deficient media the intracellular pool was completely, or almost completely, depleted of the missing nutrient. The concentrations of the other amino acids remained relatively unchanged. It is probable that arginine, a component present in very low concentration, is depleted in considerably less than 24 hours.

The absolute requirement of a nutrient for viral synthesis can only be determined when the other two variables are accurately known. The object of the nutritional studies carried out here was to investigate the relative importance of several nutrients in an effort to find that most critical in the reproduction of herpesvirus. If there was a very high specific requirement for a nutrient, omission of this from the growth medium might result in an almost immediate curtailment of viral synthesis. Cells which had been previously starved to deplete the intracellular pool should show a wider spectrum of requirements for virus growth, and thus the sensitiveness of this as an indicator of virus nutritional requirement would be diminished. For this reason experiments described here were carried out in cells that had been growing under optimum conditions.

3. Some Amino Acid Requirements for the Growth of Herpes Simplex, Vaccinia and Coxsackie B_3 Viruses

Some of the nutritional requirements for the growth of herpesvirus in RK_{13} cells were examined by comparison of the growth of the virus in complete and in various deficient media. It was found that if either arginine or histidine was omitted from the nutrient medium no virus was produced. If lysine was omitted, virus was produced to yields as high or even higher than normal. Other deficiencies which gave intermediate results will be discussed first.

Omission of all the seven water soluble vitamins and i-inositol from Eagle's medium resulted in reduced virus yield but did not inhibit virus growth completely.

The omission of cysteine was investigated to compare this with the effect found with certain enteroviruses. Rappaport (1956) found that cysteine was the amino acid of highest specific requirement for the propagation of some coxsackie and poliovirus strains. In the system studied here it was found that the cells markedly deteriorated within 48 hours in the absence of cysteine. The finding that very little herpesvirus was produced in this medium does not therefore necessarily indicate a high specific requirement of this nutrient for virus growth. Reduced virus yield may have been due to cellular damage. These results are consistent with the idea of a small intracellular pool of cysteine,

see above.

Results on the effect of the omission of glutamine indicated that this nutrient is moderately important in virus growth. This is in agreement with results obtained by Lewis and Scott (1962) and Tankersley (1964) working with different strains of herpesvirus in human cells.

When lysine was omitted from the medium it was found that the virus yield was in general slightly increased. In initial experiments with trace virus inocula the increase in virus yield was spectacular (see Table 2), and the possibility of this as a selective medium for isolation of herpesvirus was considered. After repeating the test many times it was found that, although the virus yield was increased fairly consistently, the increase was slight (see Table 4) and was not considered sufficient to outweigh the disadvantage of growing cells in a medium deficient in one of the essential nutrients.

Contrasting results were obtained with vaccinia virus growing in RK_{13} cells in lysine deficient media (see Table 19). When lysine was omitted from the growth medium there was almost a 1000-fold reduction in virus yield. Coxsackie B_3 virus growing in HeLa cells gave an intermediate result. There was some plaque reduction but still considerable virus growth (see Table 20).

A comparison of the growth of herpesvirus and vaccinia virus in RK_{13} cells in lysine-free medium suggests that there is a low demand by herpesvirus for this nutrient and a considerably higher demand by vaccinia virus. This does not give any indication of the magnitude of the intracellular pool of free lysine. In this case, and as will be considered later relative to the arginine studies, it is not possible to guess whether nutritional requirement reflects the composition of general virus induced protein, or whether it is related to the amino acid composition of the protein of the actual virus particle.

Growth of herpesvirus in a histidine-free medium was measured. It was found that in the absence of this amino acid, production of new infective virus ceased, but there was considerable development of viral CPE. One possible explanation for this is incomplete virus growth. Virus entering deficient cells may proceed along the synthetic pathway at least to the stage of producing CPE but not so far as to produce mature virus. Munk and Sauer (1964) have shown that syncytical formation by herpesvirus can occur without replication of virus nucleic acid. Since the virus yield was so much less than 1 pl.f.u. per cell (see Table 2) it is unlikely that the CPE developed with a reduced complement of mature virus.

Omission of arginine from the growth medium of herpesvirus-infected RK_{13} cells produced interesting results. In the absence of this nutrient virus production and viral CPE stopped. Similar results were obtained by Tankersley (1964) in his studies on herpesvirus-infected human cells. For the 48 hour duration of preliminary tests no detrimental effect on the cells due to nutrient deficiency was observed. It appeared that this amino acid was of high specific requirement. Without any period of prior starvation, omission of arginine from the growth medium resulted in the inability of cells to support one complete replication cycle of herpesvirus (see Figure ii).

Vaccinia-infected RK_{13} cells growing in an arginine-free medium, however, were able to produce some new mature vaccinia virus (see Table 16). The yield was greatly reduced but the fact that some vaccinia was produced indicates that the viral synthetic mechanisms were able to operate. Similarly with coxsackie B_3 virus in HeLa cells, although there was a reduction in plaque formation considerable virus growth did occur (see Table 20). These findings, particularly with RK_{13} cells, suggest not only that the arginine pool is small but moreover that the requirement of arginine for herpesvirus replication is greater than that for vaccinia replication. It probably was greater too than that for coxsackie B_3 although this was investigated in

another cell line.

Whether the high requirement for arginine in herpesvirus replication indicates a high arginine content in the virus particle or whether, more generally, a high arginine content in virus induced protein, is a question which cannot be resolved at present. It would be interesting to compare these findings with an amino acid analysis of the respective virus particles and also to see how the requirement for basic amino acid varies with the surface electrostatic charge of the virus.

4. A Tissue Culture Model of a Latent Infection with Herpes Simplex Virus

In the development of a tissue culture model of a latent herpetic infection by nutritional imbalance, it seemed that the effect of an arginine deficiency was worthy of further study. It was found that in arginine-free medium, RK_{13} cells remained morphologically normal and even multiplied slowly for 3-4 days (see Table 6). When cells were infected with herpesvirus under these conditions the virus was unable to complete a single replication cycle of normal duration of 8-9 hours. Thus it would appear that the need for arginine in viral synthesis is greater than that for normal cellular metabolism. This can be envisaged as a concentrated series of demands for the materials of protein synthesis made at the initiation of the viral synthetic processes, as compared with the slow continual demand made to maintain normal cellular metabolism particularly in the absence of active cell division.

It was clearly shown in many experiments (e.g. see Figure ix) that the replication cycle of herpesvirus was resumed when arginine was replaced in the medium of cells carrying suppressed virus. Thus a system with the essential characteristic of a latent infection, viz., suppression of infection and subsequent restimulation of viral activity, had been achieved. The attempt was then made to manipulate the concentration of arginine in

the medium and hence in the intracellular pool so that cell survival was prolonged but virus was still unable to reproduce.

It was shown (Table 6) that RK_{13} cells did not grow well in calf serum-free medium; a large proportion of cells died after 3-4 days growth. Morgan et al. (1950) found that a number of other cell types did not grow well in serum-free medium. Merchant et al. (1962) have suggested that methylcellulose could substitute to some extent for the albumins and other large molecules in serum which exert a 'cushioning effect' against osmotic changes. This was found not to be so in the system tested here; methylcellulose was unable to protect the cells against the adverse effects resulting from serum omission.

Herpesvirus growth in arginine-free medium supplemented with various concentrations of calf serum was investigated for the following reasons. Firstly, as stated, cells did not grow well in the absence of calf serum. Secondly, since the aim of the laboratory model was to imitate a phenomenon naturally occurring in the whole animal, it was important to know if herpesvirus could be suppressed by an arginine deficiency in the presence of serum. The calf serum used was undialysed and so would contain trace amounts of arginine (Eagle et al., 1957).

Survival of cells in arginine-free medium was improved when calf serum at a concentration of 5 or 10 per cent. was incorporated. Cells were grown for up to 19 days, including one subculture, in arginine-free medium supplemented with 5 per cent. calf serum. However, optimal growth was never achieved and always the cultures eventually died. Normally these cells are grown in calf serum supplemented 199 and even in complete Eagle's medium containing calf serum they never grew as well as usual. Experiments were carried out in modified Eagle's media because of the very complicated composition of medium 199.

The effect of a range of concentrations of calf serum in arginine-free medium on the growth of herpesvirus in RK_{13} cells was investigated. It was found at low multiplicities of infection, that up to 20 per cent. calf serum could be added and still leave the medium insufficient for the replication of herpesvirus (see Table 11). When cells were infected at high multiplicities (see Figure iv), some virus was produced in the 20 per cent. calf serum medium. This was still only about one third of the yield obtained in normal Eagle's with no calf serum. It was found in these cultures, however, that even in the presence of 5 per cent. calf serum slight viral CPE did develop slowly.

The following are two possible explanations of these results. Firstly, it may be that at high

multiplicities of infection some cells are killed, and in dying liberate arginine and other nutrients for surviving infected cells, analagous to the finding of Stoker and Sussman (1965) that feeder layers of cells can make good an inositol deficiency. Secondly, the small amount of arginine in the calf serum may support viral replication to the stage of producing CPE but not as far as the production of mature virus. The stage at which the deficiency of arginine blocks the replication cycle is discussed later. Both these mechanisms may operate simultaneously to some extent.

Vaccinia virus growing in RK_{13} cells in serum supplemented arginine-free medium was found to attain maximum yield in the 20 per cent. serum medium (see Table 16). Comparable yields were obtained from infected cells growing in serum-free Eagle's medium and in arginine-free medium containing between 5 and 10 per cent. calf serum. The results with HeLa cells and coxsackie B_3 virus showed that plaque counts obtained in Eagle's medium could be obtained in arginine-free medium containing between 1 and 5 per cent. calf serum. Analysis by paper chromatography indicated that a one per cent. solution of calf serum contained about as much arginine as a normal Eagle's medium. This varied slightly with the batch of calf serum. The collected results on calf serum analyses are summarised in Table 26.

Table 26. The assay of arginine-like material in calf serum.

Assay system	Comparable concentrations of 'arginine' were found in:
Herpesvirus in RK_{13} cells	$\frac{N}{3}$. Eagle's* and 20 per cent. CS Arg^-
Vaccinia in RK_{13} cells	Eagle's and 5-10 per cent. CS Arg^-
Coxsackie B_3 in HeLa cells	Eagle's and 1-5 per cent. CS Arg^-
Paper chromatography	Eagle's and ~1 per cent. CS Arg^-

'Arginine' = material that replaced arginine in virus growth or that moved as expected of arginine in a paper chromatogram.

CS Arg^- = calf serum, arginine-free medium.

* The virus yield obtained in 20 per cent. CS Arg^- was only one third of that expected from Eagle's medium.

It was found with all three viruses growing in complete medium, that plaque formation and virus yield increased as the concentration of serum in the medium increased. The one exception to this was vaccinia virus growing in cells maintained in a 20 per cent. calf serum Eagle's medium when the virus yield was less than that obtained in a 10 per cent. calf serum medium. Plaque formation and virus yield in deficient media, when this occurred, increased with increasing serum concentration.

It was also seen that for herpesvirus-infected RK_{13} cells growing in media containing increasing concentrations of arginine, the virus yield did not increase greater than

twice normal (see Figure v). Therefore, as would be expected, the beneficial effect of calf serum was not restricted to increasing the arginine concentration in the medium. It is thus difficult to analyse exactly the effect of adding calf serum to an arginine-free medium. The interesting fact is that herpesvirus-infected cells in arginine deficient medium seemed unable to use the nutrients supplied in the serum to supplement the deficiency. A similar deficiency in vaccinia or coxsackie B_3 infected cells was overcome by comparatively low concentrations of serum. Coxsackie B_3 virus growing in HeLa cells was almost as sensitive an assay system for arginine in calf serum as was two-dimensional paper chromatography (see Table 26). These facts suggest the possibility that a nutritional deficiency in herpesvirus-infected cells <u>in vivo</u> could arise more easily than with some other viruses since the deficiency would be less likely to be masked by the presence of serum. If this were so the possibility of a latent infection with herpesvirus arising from nutritional imbalance is conceivable.

Since experiments indicated that even at high multiplicities of infection, virus growth was still inhibited in arginine-free medium containing 5 per cent. calf serum, this was investigated further as a tissue culture model of a latent infection. Cells survived in

this medium for about 19 days and decreased progressively in size. The duration of this type of viral suppression or latency was investigated as well as the conditions required to reactivate or 'recall' latent virus.

The survival of latent viruses as infective foci was investigated. These experiments depended on the facts that supernates from latent cultures contained no infective virus, from the results illustrated in Figure i, and also that herpesvirus-infected cells did not divide (Wildy et al., 1961). For these reasons an infected cell in a monolayer maintained in a deficient medium could not give rise to secondary foci of infection. When complete medium was replaced on the latent culture a primary site of infection was either stimulated to form a plaque or not. Failure to form a plaque may have been due to failure of the virus to recover or else to loss or death of the cell.

Results shown in Table 10 indicate that the period required for a latent virus to recover and induce formation of a plaque was constant as long as the cells in the population had not deteriorated. The number of infective foci, however, decreased as the duration of suppression was extended. The results shown in Table 13 are similar for the first few days of suppression. This decrease in the number of recoverable infective foci was unlikely to be due to the inability of suppressed virus

to resume metabolism, since it has been shown that some latently infected cells could be restimulated to virus production after about 20 days. However, the possibility of cells recovering spontaneously from the virus infection cannot be eliminated. The more likely explanation is that some infected cells die and drop off the glass. There must be a certain turnover of cells in a monolayer, with some dying and others growing to replace them. Infected cells are as likely to die as normal cells, if not more so.

However, when the duration of suppression was extended beyond 7 days (see Table 13) and the cells began to diminish in size, the period in complete medium required for recall of virus and subsequent plaque formation gradually increased. After 16 days suppression, 6 days in complete medium were required to form lesions of the size normally attained in 3 days. This may have been due to the depletion of cellular enzymes and a slowing down of the metabolic processes required for initiation of viral replication caused by nutritional inadequacy. If cells could be maintained with the minimum essential concentration of arginine it is possible that this extension of the viral recovery period could be avoided.

After 21 days suppression, viral CPE developed without the addition of complete medium. This spontaneous virus outgrowth is probably due to the release

of arginine and other nutrients from those cells which died first as the result of prolonged nutritional imbalance. It was seen in these cultures that about one half of the cell sheet disintegrated with general cell deterioration but without development of viral CPE.

Tankersley (1964) had reported some experiments on the recovery of herpesvirus after an arginine deficiency induced suppression. These experiments were carried out in tube cultures and virus activity was scored as CPE and virus yield per tube. The maximum duration of suppression was 24 hours. He reports that virus was increasingly difficult to recover with increasing duration of suppression. This does not appear to be the case in the system investigated here when the suppression is of a comparatively short duration, viz., up to a few days. The differences between these results may have been because Tankersley was observing CPE rather than individual plaques. The increase in the lag before virus recovery might be explained on the basis that the number of surviving infective foci decreases and consequently the time for extensive CPE to develop increases. The time required for an individual focus of infection to recover did not significantly increase after short periods of suppression.

Fine control of nutrient balance appears to be a promising approach in the development of an in vitro

model of a latent herpesvirus infection. The system investigated here perhaps could be improved by adding low closely defined concentrations of arginine and working with dialysed calf serum. It would of course be optimal to carry out experiments in cells maintained in variants of their normal (199) growth medium. Variations on the concentrations of other nutrients could be investigated simultaneously. For example there is obviously a high requirement for amino acids other than arginine and histidine since it was shown (see Table 2) that Hanks BSS supplemented with glucose as energy source, and containing arginine and histidine, was unable to support the replication of herpesvirus. By careful balance of other nutrients, perhaps reduced arginine and histidine with increased lysine, a medium may be developed that is adequate for normal cell metabolism but insufficient for virus growth. These considerations need not be restricted to the essential amino acids but extended to include all nutritional requirements. Results recorded in Table 5 indicate that herpesvirus grows considerably better in the presence of added adenine and/or glycine. Conversely it is possible that the restriction of the metabolism of these compounds might result in a depression of virus growth.

On the basis of these studies it appears that imbalance of nutrients as a cause of latency in herpes-

virus infection in the whole animal is a distinct possibility. Slight changes in the composition of the supply of nutrients available to the cells may control whether or not the infecting Herpes simplex virus will proliferate. Arginine appears to be a factor of principal importance but many other nutrients may exert similar although lesser effects. The fact that calf serum was unable to substitute for the missing nutrient in tissue culture experiments with herpesvirus supports the suggestion that conditions inadequate for the growth of the virus, could be established in vivo.

The hypothesis of the establishment of latency by nutritional imbalance is not inconsistent with the other broader explanations of this phenomenon. Such factors as fever, stress, chilling, or hormonal imbalance, etc., may result in a disturbance of the balance of the components of the interstitial fluids and thus cause either an eruption or suppression of the activity of Herpes simplex virus.

5. Selective Media for Virus Isolation

The investigation of the specificity of nutritional requirements for the growth of herpesvirus, vaccinia and coxsackie B_3, and in particular the demonstration of the effect of arginine concentration on herpesvirus growth (see Figure v), have stressed the importance of the composition of the nutrient medium on the growth of any particular virus. To approach optimum conditions for virus isolation, media should be developed which are tailored to the requirements of the virus being sought. Optimum media would vary with the virus under study and the type of culture cells being used. The idea of using such a large number of specific media in a routine diagnostic laboratory is not feasible. However, in a survey concerned with only a small number of virus types it would be profitable to precede attempted isolations by a detailed study of the nutritional requirements of the virus subjects of the survey and to develop culture media accordingly. Such results would be greatly affected by the presence or absence of contaminants in the tissue culture cell line, particularly of carried viruses or pleuropneumonia-like organisms (PPLO). As indicated by Kenny and Pollock (1963) PPLO specifically deplete arginine stores from the medium and this alone would have a marked effect on the success or failure of herpesvirus isolation.

6. The Fate of the Herpesvirus Particle in an Arginine Deficient Cell

A series of experiments was carried out in an effort to find out why cells deficient in arginine were unable to support the replication of herpesvirus and if possible to find how far the virus replication cycle progressed before being stopped. In RK_{13} cells maintained in arginine-free medium, herpesvirus was found to attach at the normal rate (see Table 9). Growth curve experiments (see Figure ii) showed that the virus penetrated at the normal rate and that infectivity was eclipsed as usual. Attachment, penetration and entry into physical eclipse have been clearly demonstrated in a series of electron micrographs of ultra-thin sections of infected cells growing in complete and in arginine-free medium. There was no observable decrease in the rate of these processes in deficient medium.

Tests were carried out to investigate the development of virus-induced macromolecules, virus particles and cytopathic changes. In infected cells growing in arginine-free medium no virus-induced protein could be observed by the complement fixation test or by staining with fluorescent antibody. Here and in all other tests controls of infected cells growing in complete medium were included for comparison; these will be referred to as normal infected cells. Normal infected cells had

developed complement fixing antigen and also viral protein detectable by the fluorescent antibody staining technique within 24 hours.

Characteristic changes occur in the nuclei of herpesvirus infected cells. Nucleoli disappear with concomitant margination of the chromatin (Love and Wildy, 1963). It has been shown by autoradiographic studies, carried out by Munk and Sauer (1964), that viral nucleic acid synthesis occurs in the inner part of the nucleus and not in the marginated chromatin. Nuclei of infected cells were examined after staining with acridine orange and it was found that no such changes occurred in infected cells from arginine-free medium while those from complete medium exhibited the characteristic changes (see Figure vii (a-d)). Sometimes although infrequently, formation of small syncytia was observed in infected cells growing in arginine-free medium. An example of this is shown in Figure vii (f). This may be due to partial virus growth in cells with larger than normal arginine pools. Nuclei in the electron micrographs were also examined and showed similar results. The absence of nuclear changes in latent cultures suggests that no extensive viral DNA synthesis had occurred.

Cells were also examined for intracellular viral

lesions, formation of complete or incomplete virus particles and general cytopathic effect. In the light of the above findings negative results may be expected from these tests. Giemsa staining of infected cells showed the typical Cowdray type A inclusions in normal infected cells while no inclusions were observed in the latently infected cells. In a detailed examination for viral inclusions and virus particles samples of infected cells, which had been grown in complete and deficient media, were examined by electron microscopy. Cells from both cultures appeared similar for the first 6 hours. In the 9 hour samples, new virus appeared in the nuclei of some normal infected cells but none in the nuclei of the deficient cells. At 11 hours most of the normal infected cells contained virus particles in the nuclei; none was observed in suppressed cultures.

Herpesvirus does not develop in the nucleus in crystalline array as does adenovirus (Morgan et al., 1956) or as does reovirus (Dales, 1963) in the cytoplasm. Virus appeared almost randomly distributed throughout the nucleus. Watson et al. (1964) have suggested that virus particles develop from masses of amorphous material in the nucleus. Particles were sometimes seen around dark staining masses (see Figure viii (i) v and (k) v), but by no means consistently.

In a preliminary note, Chitwood and Bracken (1964) described the replication of Herpes simplex virus in a

very elegant series of electromicrographs. These workers inhibited the replication processes of herpesvirus using mixtures of p-fluorophenylalanine and phenylalanine. They present photographs which they interpret as showing the emergence and assembly of viral sub-units to form virus particles. The material described as viral sub-units lies in the nucleus in crystalline array and is surrounded by what are undoubtedly herpesvirus particles similarly aligned. A somewhat similar, although less spectacular, distribution of virus particles is shown in Figure viii (n). This is a record of a natural chance phenomenon and lends support to the explanation of the development of herpesvirus offered by Chitwood and Bracken.

Later in the series of normal infected cells, fusion of cells to form syncytia was observed. Virus particles were also observed leaking out of the nucleus. As particles passed from the nucleus many were observed to acquire an outer envelope which appeared to be derived from the nuclear membrane (see Figure viii (l)). Particles in the cytoplasm were seen close to the nucleus, others further from the nucleus and some close to the cytoplasmic membrane. In these cells the characteristic disappearance of the nucleoli and margination of the chromatin occurred. No such changes were observed in infected cells from the deficient medium even after 23 hours' incubation. No nuclear changes,

no inclusions and no virus particles complete or incomplete were seen in infected cells which had been grown in arginine-free medium.

Experiments were carried out to determine how far, in terms of time, the virus replication cycle proceeded in arginine deficient cells before it was stopped due to insufficiency of arginine. As previously considered this very probably would be affected by the duration of the starvation period before the cells were infected. All experiments, unless specifically stated otherwise, were concerned with infection of optimally metabolising cells with no previous history of nutritional imbalance. As shown in Figure ix an attempt was made to measure the lag phase after complete medium was added to suppressed cells before infective virus was formed. By a comparison of the duration of the lag with the duration of the normal eclipse phase it was hoped to get an indication of the distance travelled along the viral replication pathway before the breakdown occurred. If, for example, the lag phase in growth curve C (Figure ix) was only one third the duration of the normal eclipse phase this would suggest that two thirds of the replication cycle had been completed before the block occurred. These measurements are in terms of time and only give an indirect indication of how far growth has progressed in biochemical terms.

The normal growth curve (A Figure ix) and results obtained in several other experiments indicate that about 8 hours is required for one complete growth cycle of this strain of herpesvirus in RK_{13} cells. Due to the asynchrony of the processes in infected cells in a population, the maximum yield in a one step growth curve is not achieved until between 18 and 24 hours post-infection. Infected cells which had been maintained in arginine-free medium for 24 hours were stimulated to produce virus by addition of complete medium and continued incubation. The growth curve obtained after stimulation, C Figure ix, showed that new virus was produced between 8 and 12 hours after the addition of complete medium. Repeat experiments of this type indicated that the first appearance of new virus occurred about 9 hours after the addition of complete medium. This suggested that there was no reduction in the duration of the eclipse phase in infected cells that had been incubated for 24 hours in arginine-free medium.

However, it was possible that part of the delay before the production of new virus in C Figure ix was due to the adverse effect of arginine starvation on the cells. Some time may have been required for the added arginine to penetrate the cells and for the cells to replenish the stock of enzymes necessary for the initial stages of the virus growth cycle. The latter is a more distinct possibility since in general amino acids are absorbed

fairly quickly into cells and such penetration would account for no more than a few minutes delay.

To measure any such delay, growth curve experiments were carried out on cells which had been previously incubated for 24 hours in complete medium and on others previously incubated in arginine-free medium. The results are shown in Figure x, and indicate that cells previously incubated in arginine-free medium did not produce as high a final yield of virus as normal. This may have been due to a difference in the numbers of cells which were inoculated with virus. In the deficient medium more cells may have died or else fewer may have multiplied than in the complete medium. This reflects the findings shown in Figure ix, although there the difference is more marked. A greater difference was not unexpected since, in addition to the explanations suggested above, there was the added possibility of infected cells dying in the experiment illustrated in Figure ix. Death of infected cells has been discussed and is illustrated by results shown in Tables 10 and 13.

As well as the reduced final yield in the 'prior starvation' experiment illustrated in Figure x there was a delay in the first appearance of new virus from cells that had been pre-incubated in the absence of arginine. A comparison of the two curves in Figure x suggests that previously starved cells take about 2 hours longer to produce virus than do normal cells. If this is applied

to the results shown in Figure ix it would suggest that the length of the lag phase required after restimulation, curve C, is 2 hours less than the previously determined 9-12 hours, that is, 7-10 hours is required for virus production. The normal growth cycle takes 8 hours for completion and therefore, at most, virus replication had continued for only 1 hour before stoppage occurred due to shortage of arginine. Since it is so difficult to interpret growth curves precisely, because of the asynchrony of the cultures, the above times are not exact; but it is fair to say the blockage due to shortage of arginine comes very early in the replication cycle, almost certainly within the first 2 hours.

All the findings on the fate of a herpesvirus particle infecting a cell in arginine-free medium can now be gathered. The virus absorbs and penetrates with subsequent eclipse of infectivity and of particulate structure. As far as it has been possible to determine there is no synthesis of virus-induced protein or of viral DNA. However it must be noted that the most sensitive techniques for the detection of such macromolecules could not be employed in these studies. No viral inclusions or virus particles develop. There are no cytopathic changes in the infected cell, neither nuclear changes nor formation of syncytia. Timed studies suggest that only about 1 hour of the 8 hour

replication cycle is completed before the block occurs.

In a recent paper on macromolecular synthesis in cells infected with Herpes simplex virus, Roizman et al. (1965) showed that the rate of protein synthesis in infected cells decreased for the first 3 hours after infection and that the rate of decrease varied with multiplicity of infection. Between 3 and 6 hours post-inoculation the rate of uptake of amino acids was stimulated; thereafter the rate declined. This suggests that viral synthesis is affected by the multiplicity of infection, to some extent. It may also suggest that the bulk of viral protein synthesis occurs between 3 and 6 hours post-inoculation. Therefore breakdown of the virus replication cycle due to insufficiency of an essential amino acid might be expected to occur within the first 3 hours after inoculation.

Russell et al. (1964), in a study on the replication of the HFEM strain of Herpes simplex virus, have shown that complement fixing antigens start increasing 1-2 hours post-infection. Some enzymes concerned with nucleic acid synthesis started increasing at 2-3 hours and viral DNA at 5 hours post-infection. Virus particles started appearing between 3 and 9 hours and infective virus at 5 hours. The replication cycle of this virus-cell system is, therefore, shorter than that studied here. However, even with the HFEM strain of

virus, most of the viral protein synthetic processes appear to start 2-3 hours after inoculation. This is in general agreement with the findings of Roizman et al. (1965), using the MP strain of Herpes simplex virus. It is also consistent with the conclusion drawn here that the virus replication cycle breaks down, in the absence of an essential amino acid, 1 or at most 2 hours after infection.

A possible interpretation of these results is that virus particles penetrate and are dissociated within the cell by the action of cellular 'stripping' or 'uncoating' enzymes analogous to those postulated by Joklik (1964) in poxvirus replication. It may be depletion or destruction of some of these enzymes in arginine starved cells that accounts for the delay in virus maturation shown in Figure x. The small intracellular pool of arginine is insufficient to support the synthesis of virus-induced proteins. For individual amino acids there is found a threshold concentration necessary to sustain protein synthesis and cellular growth (Eagle et al., 1961). Synthesis of the large battery of enzymes and other proteins necessary for the initiation of replication of any infecting virus would put a heavy strain on the arginine pool. The threshold concentration of arginine necessary for the synthesis of herpesvirus proteins appears to be particularly high and the intracellular pool of arginine low, so that little or no synthesis

occurs.

Some form of virus 'blueprint' must remain within the cell since growth can be reactivated even after long periods, but whether this exists in the nucleus and in what state it has been impossible to tell. It is, however, singularly stable since restimulation of virus reproduction has been demonstrated as long as the cells containing virus remain viable. One possibility is that the virus may react with the cell chromosome and remain latent in some form of nuclear union. If the arginine findings could be repeated in diploid cells it would be interesting to look for chromosome aberrations, as were observed in human embryonic lung and Chinese hamster lung cells after infection with Herpes simplex virus (Stich et al., 1964).

This retention of genetic information is in agreement with the synchrony or relative synchrony of virus production observed in Figure ix. The time required for virus particles to penetrate cells and to reach a suitable site for initiation of viral synthesis varies from cell to cell. If some block occurred immediately after this stage but before the major synthetic processes started then those viruses penetrating and perhaps 'stripping' more slowly will have reached the same stage as those penetrating more quickly. When complete medium is added each virus-infected cell begins viral synthesis at the same time thus producing

something approaching a synchronous culture.

A method of producing synchrony in viral synthesis without the use of structural analogues or other unnatural compounds would be of great value as a tool in the biochemical analysis of the very early stages of the replication cycle. Early virus-induced enzymes and other proteins are difficult to detect, estimate and arrange in time sequence since they are produced in small quantities by a small number of cells. Later, when more cells are producing these proteins, the 'early' cells have progressed further. The culture becomes out of phase and produces a whole range of viral products thus hindering sequential analysis. If the early synthetic processes were synchronised, identification and estimation of the very first virus-induced macromolecules would be greatly facilitated.

To follow the fate of infecting herpesvirus in arginine deficient cells more exactly radio-active tracer methods as used by Roizman et al. (1965) would be of considerable help. In addition radio-active labelling of virus particles used in conjunction with autoradiography would indicate the position in the cell of the latent virus. It may also be possible to investigate this by use of ferritin-conjugated antibody techniques and electron microscopy of thin sections.

For a closer investigation of possible preliminary viral synthesis before the block induced by arginine deficiency, more elegant methods of biochemical analysis would be required, as for example those used by Keir and Gold (1963) in the identification of DNA enzymes from herpesvirus-infected cells. These methods are at present outwith the scope of this thesis.

7. A Rationale for Viral Chemotherapy

One exciting possibility arising from these findings on the high specificity for arginine in herpesvirus reproduction is the development of a chemotherapeutic agent. Since the requirement by the virus for arginine is so much greater than that of the cell it might be possible to inhibit virus synthesis relatively specifically by the use of arginine analogues. Such an analogue might become incorporated in the very early virus-induced proteins which require a lot of arginine, and it is possible that this might cause an irreversible inhibition of virus replication. Since the high demand for arginine occurs early in the growth cycle short term application of the analogue might be sufficient to inhibit virus metabolism in individual cells.

The major problem is that common to the development of any viral chemotherapeutic agent; one must kill the invading virus but spare the cell. Substitution in the cell of an essential amino acid by an analogue would ultimately result in cell death. However, infections with herpesvirus *in vivo* are often superficial. It is possible that corneal or epidermal infections could be treated by a series of short term topical applications of an arginine analogue employing a principle analogous to tyndallisation in bacteriology. Those virus particles which had reached a stage requiring much arginine might incorporate the analogue and so be

effectively eliminated; other particles nearing completion might continue development but on a second or third application of the antiviral agent progressively more viruses would be inactivated.

Arginine and herpesvirus appears a very promising combination for this line of study but it is possible that other inhibitors for other viruses could be sought in a similar way. It is hoped to investigate this possibility at a later date.

8. Interference between Herpesvirus and Vaccinia Virus

Another method used in the attempt to elucidate how the virus 'message' existed in arginine deficient infected cells was that of viral interference. The aim was to determine whether cells in arginine deficient medium carrying latent herpesvirus were able to support the replication of a second, challenge virus. Experiments were carried out, therefore, to find a virus with these two characteristics. Firstly, it should be able to grow in cells which contained insufficient arginine for the growth of herpesvirus. Secondly, it should be unable to grow in the presence of normally replicating herpesvirus.

Tyndall and Ludwig (1962) had found that coxsackie B_3 virus grew in the absence of arginine, and this finding was corroborated here (see Table 19). However, it was also shown that coxsackie B_3 virus did not grow in RK_{13} cells and since the herpesvirus-arginine studies had been carried out in these cells it was preferable to continue with the same model. Vaccinia virus was found to have a lower requirement for arginine in RK_{13} cells than did herpesvirus. At low multiplicities of infection, vaccinia grew to full yield in an arginine-free medium supplemented with 20 per cent. calf serum whereas no herpesvirus growth was detected at all in such a medium; compare Tables 11 and 15. Experiments were carried out to investigate interference between

these two viruses growing in complete medium preliminary to studies with latent herpesvirus. Later work showed (see Figure iv) that at high multiplicities of infection there was development of viral CPE and even some infective virus in herpesvirus-infected cells growing in arginine-free medium with 20 per cent. serum. This model therefore was not suitable for the original purpose. However, the results yielded basic information on the phenomenon of viral interference.

Interference of herpesvirus growth by live and killed vaccinia virus, and interference of vaccinia by herpesvirus was studied. Galasso and Sharp (1963, 1964) working with vaccinia virus and Earle's L cells showed that there was homologous inhibition with both heat-killed and ultra-violet (U/V) inactivated virus. Results presented in Table 20 indicate that there was no such homologous interference in RK_{13} cells with heat-killed vaccinia virus. It was found too, Tables 20 and 21, that heat-killed and U/V inactivated vaccinia did not interfere with herpesvirus.

Living vaccinia did interfere with the growth of herpesvirus. Interference was demonstrated using very small inocula of interfering virus thus suggesting the likelihood of an interferon mechanism. Glasgow and Habel (1962) have demonstrated a 94 per cent. plaque reduction of Herpes simplex virus by vaccinia-induced interferon whereas influenza-induced interferon had no

activity against herpesvirus. The sensitiveness of herpesvirus to the action of an interferon, produced directly or indirectly as a result of a concomitant polyoma infection, has been suggested by Glasgow and Habel (1963) to explain the maintenance of a double carrier system of cells infected with both herpesvirus and polyoma virus.

Herpes simplex virus was found to interfere with vaccinia virus, see Table 21. The interference was significant and this would have been a suitable tool for examination of latent herpesvirus infections had suitable media been available for this purpose, see above.

Henle (1950) in a review on viral interference recorded a possible interference of vaccinia by herpesvirus observed by Gildemeister and Herzberg in 1925 and 1927. There is no record of interference of herpesvirus by vaccinia, and no definite record of interference of vaccinia by herpesvirus in any of the principal reviews (Henle, 1950; Schlesinger, 1959; and Wagner, 1960). Indeed Syverton and Berry (1947) observed cytoplasmic and intranuclear inclusion bodies in the same cell in cultures doubly infected with herpesvirus and vaccinia virus and deduced that both viruses could proliferate simultaneously in the same cells.

The results presented here demonstrate that, at least with the virus strains used and RK_{13} cells, vaccinia and herpesvirus can interfere each with the growth of the other.

9. The Reversible Inactivation of Herpesvirus by Heparin

The second possibility investigated in this attempt to develop a tissue culture model of a latent herpesvirus infection was that of a reversible extracellular inactivation of virus. The inactivating agent considered was heparin, a sulphated mucopolysaccharide, which had been shown to have antiherpes activity (Vaheri and Cantell, 1963).

The inactivation of the HSV(MP) strain of herpesvirus is shown in Figure xii. It was found that herpesvirus was inactivated increasingly with increasing concentrations of heparin when the two were mixed before inoculation on to the cell sheet. There was, however, a resistant fraction of virus in all preparations tested.

The mode of action of heparin as an antiherpes agent has been studied in some detail by Vaheri (1964). He suggests, on the basis of a convincing series of experiments, that heparin acts on a very early stage in the virus-cell interaction, probably by preventing the initial electrostatic attachment of the virus. This may be effected by the action of heparin, a polyanionic molecule, on either the surface structures of the virus particles, the host cells, or by the combined action on both. The experiments recorded here with HSV(MP) and RK_{13} cells are in general agreement with those of Vaheri, who used another herpesvirus strain and human

amnion cells. However, it was found here that higher concentrations of heparin were required for virus inactivation and that, in every case, the fraction of resistant virus was considerably higher.

If the action of heparin is to prevent electrostatic bonding between virus and cells, and this seems likely, then the proportion of enveloped and naked herpesvirus particles in the inoculum may affect the resistance or susceptibility to heparin. The outer membrane of enveloped particles has been found to be serologically related to the host cell membrane (Watson et al., 1963). Therefore, heparin may act only on naked particles, or only on enveloped particles and then perhaps also on the host cells. In each case there may be a resistant fraction unaffected by the polyanion. The stock herpesvirus preparation used here may have differed significantly from that of Vaheri in these relative proportions.

The results of experiments on the effect of heparin on vaccinia virus when the two were mixed before inoculation on to the cell monolayers are shown in Figure xii. Up to very high concentrations of heparin (1,000 µg per ml) there was no virus inactivating effect, rather there was a protective effect on the virus. This was similar to the result found when vaccinia was mixed with homogenates of cells prior to inoculation on to cell cultures (see Part I). If this was analogous it was

probably due to prevention of virus loss through adsorption on to glass surfaces.

The effect on herpesvirus plaque reduction of heparin when it was added 3 hours after virus inoculation is shown in Figure xiii. Two curves were drawn to describe the antiviral effect. Curve A shows the percentage plaque reduction based on a heparin-free control which contained Methocel. No secondary plaques developed in the presence of Methocel and therefore the reduction on heparin cultures could have been a measure of the effect of heparin on virus not yet penetrated and on intracellular virus. Curve B based on the heparin-free Methocel-free control shows plaque reduction due to elimination of secondary plaque formation, and the possible effect of heparin on virus not yet penetrated and on intracellular virus. Reduction in B was greater than that in A. Greater reduction than shown by either of these was obtained by admixture of virus and heparin before inoculation.

When these results are considered together they suggest that the effect of heparin is mainly on extracellular virus and to some extent on virus adsorbed but not fully penetrated. This supports the postulation, made by Nahmias and Kibrick (1964) and Vaheri (1964), that the effect is on the initial electrostatic bonding of virus, and indicates that heparin has little effect on the intracellular stages of viral replication.

Since heparin is a polyanion and appears to act by formation of a complex with the virus particle it would be interesting to compare sensitivity of a virus to heparin and the electrostatic charge on the virus, and to see, for example, if the sensitivity of herpesvirus corresponded with a significant positive charge on the virus particle, or else to see if the sensitivity varied with the cation concentration in the medium.

Surprising results were obtained when heparin was added to vaccinia infected cultures 3 hours post-inoculation; these are shown in Figure xiv. Intracellular vaccinia was susceptible to the action of heparin. These results may be explained by postulating that, while extracellular vaccinia is insusceptible to the action of heparin, some viral component which becomes exposed inside the cell is sensitive to heparin or it may be that some cellular processes concerned in initiation of infection are affected. Other workers, e.g. Vaheri (1964), have found vaccinia insensitive to heparin in <u>in vitro</u> experiments but only extracellular activity was considered.

The <u>in vivo</u> susceptibility of two other poxviruses has already been described. Thiery (1953) reported that heparin could inhibit infection with myxoma virus, and more recently Higginbotham and Murillo (1965) reported on the influence of heparin on the resistance

of rabbits to infection with fibroma virus. It has been found that the growth of fibrona-induced tumours was not affected by specific antiserum (Hurst, 1964) whereas Higginbotham and Murillo (1965) found that heparin could effect a reduction both in virus titre from tumours and in tumour size. The latter workers considered the mode of action of heparin as an anti-fibroma agent and favoured the explanation that it acted intracellularly. Nonetheless it is difficult to understand why poxviruses should become sensitive intracellularly while herpesvirus, which is susceptible extracellularly, should not. It is possible that infection with poxviruses increases the permeability of the cell to heparin while herpesvirus does not. This seems unlikely and is not a wholly satisfactory explanation.

Having found that heparin could inhibit HSV(MP) by a mechanism generally explained as prevention of virus attachment due to formation of a heparin complex, the dissociation of such a complex and the liberation of free virus was investigated. Two dissociation mechanisms were investigated. Firstly, simply 'diluting out' the heparin. As shown in Table 25 this results in a liberation of free virus thus confirming the findings of Nahmias and Kibrick (1964) and Vaheri (1964). The second mechanism investigated was dissociation of the

complex by the polycation, protamine sulphate. It has been shown by Nahmias and Kibrick (1964) that protamine sulphate was able to neutralise the anticoagulant effect of heparin on blood and that it was able to reduce the antiviral effect on heparin. Before the antiheparin activity was studied experiments were carried out on the antiherpes effect of protamine.

Results shown in Figure xv indicate that protamine sulphate inactivated vaccinia and herpesvirus when mixed with them before inoculation on to cells. The inactivation rates of the two viruses were the same. Since protamine was able to neutralise both viruses extracellularly while heparin neutralised only herpesvirus it would appear that the two compounds must exert their antiviral actions differently. Both appear to act early in the replication cycle since they are effective when mixed with the virus extracellularly. This has also been suggested by Vaheri (1964). If they act by formation of extracellular complexes it would seem that they act on different specific sites.

Experiments were carried out to determine the effectiveness of protamine sulphate in neutralising the antiherpes effect of heparin. Little neutralisation was observed when the two compounds were allowed to react before addition of virus. This result may have been due to the fact that it was difficult to detect any reduction of heparin activity because of concomitant

protamine antiviral activity. However, when heparin was mixed with herpesvirus, to form the postulated complex, and then protamine sulphate was added, infective herpesvirus was released from the effect of heparin. This can be visualised as firstly, formation of a herpes-heparin complex and then subsequent dissociation by the action of protamine perhaps acting because the affinity of heparin for protamine is greater than that for herpesvirus. It is difficult to see how protamine sulphate can act by dissociating virus from a heparin complex when it is ineffective in neutralising the heparin alone. As recorded above other workers have found neutralisation of heparin by protamine.

The object of this part of the work was to develop a model of a latent infection by formation of a reversible complex of the virus outside the cell. As a tissue culture model the HSV(MP) strain of herpesvirus in RK_{13} cells was not very promising. There was a large fraction, about 10 per cent., of the virus population unaffected by the presence of heparin. Moreover the heparin-virus complex is readily dissociable and this can be markedly affected by many factors. Jaques and Bell (1959) found that inorganic salts increased the dissociation of heparin-protein complexes so that ionic strength of the medium would affect the activity of heparin. Methods of reversing the activity of heparin

with release of infective virus have been demonstrated. Virus was liberated by simple dilution and by the use of the polycation, protamine. The latter was of limited practical use because of the strong antiviral action of protamine itself and the necessity to maintain a very delicate balance of concentrations between this and heparin.

The possibility of heparin being of importance as a factor disposing to the establishment of latency in an in vivo infection is more hopeful. It has been shown that heparin, a substance present in various human and animal tissues (Riley, 1963) can inactivate herpesvirus by forming a complex with it. The complex can be dissociated by diluting the heparin, by the action of an antiheparin compound, or perhaps by changes in ionic concentration of the tissue fluids. Protamine was investigated here but other naturally occurring compounds have a similar although lesser effect. Vaheri (1964) found that the inhibition of herpesvirus was antagonised by the following, in order of increasing effectiveness: serum, albumin, hyaluronidase, thrombin, and the polyamine spermine. The finding that a proportion of virus is resistant to the action of heparin would call for the postulation of a secondary antiherpes mechanism in latent states. This might be specific antiserum.

Although only speculative, the following hypothesis might be worth consideration. Heparin may act by

blocking specific sites on the host cell and on the cell derived membranes of the enveloped herpesvirus particles thus preventing virus adsorption. It is unlikely that enveloped particles act as an antigenic stimulus since they are coated with cell material. Such particles aggregate with anticell serum whereas naked particles aggregate only with antiviral serum (Watson and Wildy, 1963). It may be that the naked particles are insensitive to heparin but sensitive to the action of antiherpes serum. A delicate balance of heparin and antiserum in the tissues might result in an attainment of the latent state. An outbreak of viral activity might result if this balance was upset and some of these extracellular particles were able to penetrate a cell and initiate infection. Apart from the aspect of latency, heparin and antiherpes serum together are probably important in the localisation of infection.

Two basic approaches to the problem of latency in a viral infection have been considered. These are the possibilities of a reversible suspension of the intracellular replication cycle and of a reversible extracellular inactivation of virus. It has not been possible to decide which of these mechanisms is of primary importance and whether one alone is adequate or whether there is interdependence between the two.

Some strains of Herpes simplex virus are known to be able to spread from cell to cell; therefore, methods of extracellular virus inactivation such as by specific antiserum or heparin are probably more important in the localisation of infection rather than as causes of latency. It has been demonstrated how these agents can neutralise the virus and how, for example, an inactive heparin-virus complex can become dissociated and so permit initiation of infection. To this extent a system with the characteristics of a latent infection has been achieved. However, it is difficult to explain how the intracellular viral processes and the cell to cell spread can be stopped without the postulation of a second intracellular control mechanism. With this virus at least, the method of extracellular complex formation alone is insufficient explanation of a latent infection. It may, however, be a contributing factor and of greater importance in infections with strains of herpesvirus which involve cell-medium-cell spread.

The balance of intracellular nutrients, or more generally the regulation of cellular metabolism, has been shown to have a marked influence on herpesvirus growth. This may be one of the 'common pathways' discussed earlier, for the attainment of the latent state. Many factors could cause slight changes in the metabolism of the cell: temperature changes, slight genetic changes caused by U/V irradiation, presence of

a secondary infection and even the activity of interferon. Herpes simplex virus is critical in its requirement for at least one of the essential amino acids, arginine, and it is not impossible that a balance of nutrients could be achieved in an *in vivo* infection to cause prolonged suppression of virus but survival of cells.

Three of the significant facts about herpesvirus appear to be these:

i. the concentration of arginine required for virus growth is critical;

ii. this virus is among the very few sensitive to heparin, a substance naturally occurring in the tissues;

iii. a considerable proportion of viral progeny have a host-cell derived envelope and so are resistant to the natural immunological response of the host.

The virus can survive, activity suspended, in cells deficient in an essential nutrient. The virus can survive, reversibly bound, to heparin. The virus, or at least a considerable proportion of the virus, can side-step elimination by the normal immunological methods. It seems that these facts must somehow have a bearing on the curious ability of this virus to exist, undetected for long periods, in the host.

The latent virus, adapted as it is, to hide in the

tissues escaping total elimination by the host, must surely represent a very advanced state of evolution in the virus world. It is an interesting thought that continuous latency, with no intermittent eruption of viral activity, describes a situation analogous to that existing with the proven and postulated tumour viruses.

SUMMARY

PART I

Studies were carried out to investigate the postulated existence of specific receptor areas for poxvirus on the surfaces of susceptible mammalian cells. Before this was attempted a standard plaque titration technique was evolved for the viruses under test. The effects of using different cell lines, adsorption periods and overlay media were compared and the most suitable combination chosen as standard for use in subsequent experiments.

Inactivation of virus as a result of admixture with tissue cell homogenate was selected as indicative of specific receptor-virus union, since this was a principal criterion in similar studies by other workers. These experiments were preceded by an investigation on the general stability of vaccinia virus and subsequent findings were assessed relative to these results.

No inactivation of either of two strains of vaccinia virus or of cowpox virus by homogenates of susceptible tissue cells could be demonstrated; rather an increase in virus titre was consistently observed. Two sets of results served to confirm that the experimental model was not at fault. Inactivation of poliovirus by homogenates of susceptible but not of insusceptible cells was demonstrated. Attachment and subsequent eclipse of infectivity of vaccinia virus

resulted when homogenates were replaced in the test system by viable cells.

The cellular location and nature of the protective material in the homogenate were investigated. It was found to be dispersed throughout the cell and was not of a highly specific protein or carbohydrate configuration. The following possible modes of action were considered: protection of virus against heat inactivation, viral aggregation or adsorption to glass. The main effect of the homogenates was found to be prevention of virus loss by adsorption to glass. As the glass surface area increased, virus loss increased. In the presence of homogenate, virus survival was constant regardless of the glass surface area.

The failure of tissue cell homogenates to cause viral inactivation does not totally eliminate the possibility of there being specific receptor sites for these viruses on cells. However evidence collected from this, and other sources, supports the hypothesis that there is a non-specific mode of attachment of poxviruses to cells.

PART II

The object of these studies was to develop a tissue culture model of a latent infection with Herpes simplex virus (MP). The principal attempt to achieve this was

by variation in the balance of nutrients available to infected cells. The possible attainment of the latent state by a reversible extracellular inactivation was also considered.

1. Earlier studies by other workers on the nutritional requirements for cell and virus growth have been reviewed so that the present work can be considered relative to this.

2. The effect of omission of certain amino acids and vitamins from the growth medium of herpesvirus-infected cells was investigated in some detail. In addition some omissions were studied relative to the growth of vaccinia and coxsackie B_3 viruses.

The two amino acids studied in most detail were arginine and lysine. When arginine was omitted from the medium herpesvirus replication was arrested, vaccinia virus production was markedly reduced but coxsackie B_3 virus production was only slightly depressed. Omission of lysine from the medium of herpesvirus-infected cells caused no reduction in virus yield, whereas vaccinia-infected cells produced only trace amounts of virus. Coxsackie B_3 virus gave an intermediate result.

3. It was found that replacement of arginine in the medium of infected cells after a period of deprivation resulted in a resumption of the growth of herpesvirus and a restimulation of the growth of vaccinia virus.

Manipulation of the arginine content of the medium as a means of establishing a latent herpesvirus infection in tissue culture was considered.

The effect of adding undialysed calf serum to uninfected and to herpesvirus-infected cells in arginine-free medium was studied. By this means the survival of uninfected cells was improved. Medium containing 10 per cent. calf serum, however, was still inadequate for herpesvirus replication. Infected cells were maintained for over two weeks in arginine deficient medium containing 5 per cent. calf serum without production of virus. Throughout this period virus growth could be restimulated by replacement of arginine in the medium. After 21 days' suppression the system broke down with development of viral CPE and death of uninfected cells as a result of prolonged nutritional imbalance.

Very limited herpesvirus growth occurred in arginine-free medium supplemented with even 20 per cent. calf serum, and then only when cells were infected at high multiplicities. However, comparatively low concentrations of calf serum could be substituted for arginine in the growth of vaccinia or coxsackie B_3 viruses.

4. Herpesvirus yield was increased when the content of free arginine in the medium was increased. Although the final yield was never greater than twice normal, the variation was sufficiently marked to stress the

importance of the development of selective media for virus isolation.

5. An attempt was made to elucidate the fate of the herpesvirus particle inside an arginine-deficient cell. The virus was able to adsorb, penetrate and enter eclipse as usual; but it was not able to undergo one complete replication cycle. Little or no synthesis of viral or virus-induced protein, or of viral nucleic acid, occurred. There was no development of viral inclusions, complete or incomplete virus particles or viral cytopathic effect. Timed studies indicated that only about one hour of the replication cycle was completed before stoppage due to shortage of arginine. Replacement of arginine in the medium resulted in an almost synchronous resumption of viral replication.

6. Because of the highly specific requirement for arginine in herpesvirus replication it may be possible to develop arginine analogues as inhibitors of herpesvirus growth.

7. The susceptibility of this strain of herpesvirus to the action of heparin and the possible development of a latent infection by reversible extracellular neutralisation of virus was considered.

Inactivation was demonstrated when the virus and heparin were allowed to react extracellularly. As the virus penetrated the cell the effectiveness of the heparin diminished. The reversal of herpesvirus

inhibition by heparin was demonstrated either by diluting out the heparin or by addition of the anti-heparin agent, protamine sulphate. However, in all preparations tested, there was a fraction of virus resistant to the action of heparin and therefore, this system was not suitable for the development of an *in vitro* model of a latent infection.

An investigation of the sensitivity of vaccinia virus to heparin was included for comparison. Heparin was not active against vaccinia when the two were allowed to react extracellularly; but after the virus had penetrated the cell it became susceptible to the neutralising effect of heparin.

8. Some interference experiments were carried out as part of the investigation of the latent state induced by an arginine-deficiency. It has been possible to demonstrate interference of herpesvirus growth by live vaccinia virus. An interferon mechanism may be operative. Interference of vaccinia virus growth by herpesvirus was also recorded.

9. The relative importance of reversible intra- and extracellular inactivation of virus in the attainment of the latent state *in vivo* has been considered. Balance of nutrients may be one of the main factors controlling the fate of an infecting virus, but it is possible that a reversible extracellular neutralisation of virus is a contributing factor.

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Publication from the contents of this thesis

Statement in accordance with Ph.D. additional regulation No. 13:

A paper by the author entitled 'Nutritional Requirements for the Growth of Herpesvirus hominis in Tissue Culture', was read to a meeting of the Pathological Society of Great Britain and Ireland on July 10th, 1965.

A report of work carried out between April 1963 and January 1964 was submitted to the National Fund for Research into Poliomyelitis and Other Crippling Diseases and appears as Appendix 22 in the Eleventh Annual Report.

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